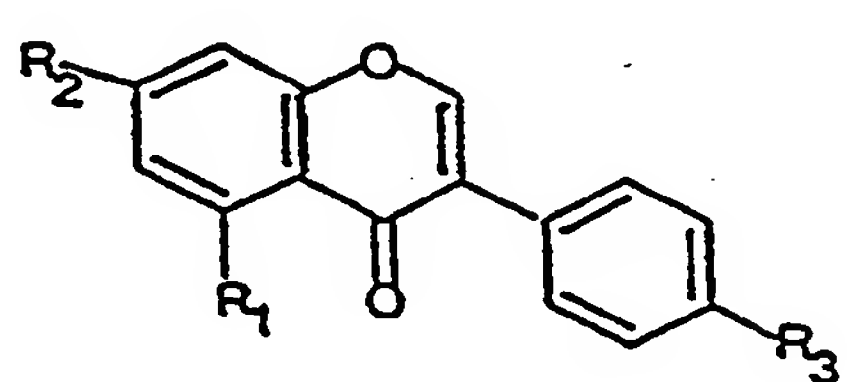




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US90/06714 <b>(22) International Filing Date:</b> 19 November 1990 (19.11.90)  <b>(30) Priority data:</b> 444,838 4 December 1989 (04.12.89) US  <b>(71) Applicant:</b> MICHIGAN STATE UNIVERSITY [US/US]; East Lansing, MI 48824 (US).  <b>(72) Inventors:</b> SAFIR, Gene, R. ; 1716 Coolidge Road, East Lansing, MI 48823 (US). NAIR, Muraleedharan, G. ; 914B Cherry Lane, East Lansing, MI 48823 (US). SI- QUEIRA, Jose, O. ; 801-207 Cherry Lane, East Lansing, MI 48823 (US).		<b>(74) Agent:</b> McLEOD, Ian, C.; 2190 Commons Parkway, Oke- mos, MI 48864 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BE (European patent), BG, BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (Eu- ropean patent), FI, FR (European patent), GB (Euro- pean patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, RO, SE (European patent), SU.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD AND COMPOSITIONS FOR STIMULATING VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI  <div style="text-align: center;"> (I)</div> <div style="text-align: right;"><b>BEST AVAILABLE COPY</b></div> <b>(57) Abstract</b> <p>A method and compositions for stimulating the growth of vesicular-arbuscular mycorrhizal fungi alone or in the presence of a plant material using isoflavonoid compounds is described. The isoflavonoids are preferably of formula (I), wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group consisting of hydrogen, hydroxyl and alkoxy, particularly methoxy.</p>		

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METHOD AND COMPOSITIONS FOR STIMULATING  
VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to the use of isoflavonoids for the stimulation of vesicular-arbuscular mycorrhizal fungi (VAM) Fungi. In particular, the present invention relates to methods and compositions which use the isoflavonoids to stimulate the growth of plant materials in the presence of the VAM fungi.

Prior Art

Several fungal species which are members of the family Endogonaceae (Zigomycetes) form endophytic symbiotic associations with roots of a large number of vascular plants in virtually all types of terrestrial habitats (Harley, J. L., and Smith, S. E., Mycorrhizal symbiosis. Academic Press, London, p.483 (1983); Safir, G. R., Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton. p. 224 (1987)). These associations are termed vesicular-arbuscular mycorrhizae (VAM) and are known to occur in at least 300,000 plant species, including most agriculturally important crops, with the exception of crucifers and a few other species. As widely known, the VAM fungi can have profound beneficial effects on plant growth, nutrition and tolerance to both abiotic and biotic stress (Powell, G. L. and Bagyaraj, D. J., VA mycorrhiza. CRC Press, Boca Raton. p.234 (1984); Safir, G. R., Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton. p. 224 (1987)). These benefits result from increased soil nutrient uptake, increased nodulation and biological nitrogen fixation in legumes, favored plant-water relationships, reduced disease severity, increased accumulation of plant growth promoting substances

and other physiological effects (Smith, S. E. and Gianinazzi-Pearson, V., Ann. Rev. Plant Physiol.. Plant Mol. Biol. 39:221-244 (1988)). Researchers and commercial firms from all over the world have recognized the great  
5 biotechnological potential for large scale application of these fungi in agriculture and forestry (Jeffries, P., Use of mycorrhizae in agriculture. Crit. Rev. Biotechnol. 5:319-357 (1987); Powell, C. L. and Bagyaraj, D. J. ed. VA mycorrhiza. CRC Press, Boca Raton. p.234 (1984); Safir, G.  
10 R., ed., Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton., p.224 (1987); and Siqueira, J. O. and Franco, A. A., Biotechnologia do solo. MEC/ABEAS, Brasilia, p234 (1988)). The use of these fungi as plant or soil inoculants could have significant impacts on agriculture  
15 and global environmental quality by reducing fertilizer and pesticide use, by reducing crop loss due to abiotic (metal toxicity, drought, adverse temperature) and biotic (nematodes and pathogens attack) stresses. In addition, these fungi have been shown to improve the survival and  
20 early growth of out plantings and increase productivity or re-vegetation in poor and disturbed sites. In fact, the outgrowth of some transplanted forest and fruit species as well as coffee seedlings is known to be improved by a range of 50 to 8000% by proper VAM inoculation. For  
25 horticultural and arable crops the reported yield increases have ranged from 5 to 290% by VAM inoculation (Powell, C. L., and Bagyaraj, D. J., ed. VA mycorrhiza. CRC Press, Boca Raton. p234 (1984); Siqueira, J. O. and Franco, A. A., Biotechnologia do solo. MEC/ABEAS, Brasilia. p235 (1988)).  
30 Considering the concerns with environmental quality in the developed nations; the high fertilizer requirement of tropical soils, where agricultural expansion is expected to happen; and the fact that global phosphate deposits will potentially be exhausted in about 70 years;  
35 there is a tremendous worldwide market for VAM inoculum.

The importance of VAM fungi for plant growth in most soils has been accepted and acknowledged by soil

chemists, agronomists, horticulturists, ecologists, farmers and nurseryman (Powell, C. L., and Bagyaraj, D. J., ed. VA mycorrhiza. CRC Press, Boca Raton. p234 (1984); Safir, G. R., ed., Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton, p224 (1987)), but current inability to supply large quantities of active and effective inoculant represents the main drawback to their large scale use.

The VAM fungi are regarded as obligate biotrophs in that they have not been successfully cultivated under axenic conditions (Hepper, C. M., VAM spore germination and hyphal growth in vitro: prospects for axenic culture. In: Proc. 7th NACOM, Sylvia et al. (ed). University of Florida, Gainesville. 172-174 (1987)); Mosse, B. Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can. J. Bot. 66:2533-2540 (1988); Siqueira, et al Can. J. Microbiol. 31:965-972 ((1985)). The lack of infective VAM fungi makes intensive studies on the basic biology, ecology, host-relationship and inoculant production technology very difficult. The culture of VAM fungi in vitro has frequently been attempted, but has met with variable results (Hepper, C. M., VAM spore germination and hyphal growth in vitro: prospects for axenic culture. In: Proc. 7th NACOM, Sylvia et al. (ed). University of Florida, Gainesville p.172-174 (1987)); Siqueira, et al. Mycologia 74:952-959 (1982); Siqueira, J. O., et al., Can. J. Microbiol. 31:965-972 (1985)). European patent application No. EP0172085 describes the axenic growth of VAM fungi; however, the growth factors are from a non-plant source and the VAM fungi would have limited ability to infect the plants. Viable spores of most species are readily germinated when plated on suitable media, but germination synchrony and the rate of germ tube growth in the absence of living roots are affected by several innate environmental and nutritional factors (Hepper, C. M., VAM spore germination and hyphal growth in vitro: prospects for axenic culture. In: Proc. 7th NACOM, Sylvia et al. (ed). University of Florida, Gainesville p. 172-174 (1987);

Siqueira, J. O. et al. Can. J. Microbiol. 31:965-972 (1985)). Although spores of most species have no specific nutritional requirement for either germination or hyphal growth, addition of certain factors has been shown to be  
5 beneficial, but only to a very limited extent (Siqueira, J. O., Can. J. Microbiol. 31:965-972 (1985)). Continued hyphal growth and sporulation are only achieved in the presence of living plant roots (Bécard, G. and Fortin, J. A., New Phytol. 108:211-218 (1988); Mosse, B., Can. J. Bot.  
10 66:2533-2540 (1988)). Japanese Patent 63-87973 (1988) showing VAM fungi inoculated with potatoes and various growth accelerators and U.S. Patent No. 4,294,037 to Mosse et al describes the growth of VAM fungi in the presence of plant roots.

15 For a long time root exudates have been thought to play an important role in the initiation and extent of VAM formation (Harley, J. O., and Smith, S.E., Mycorrhizal symbiosis. Academic Press, London. p.483 (1983)). The presence of roots stimulates hyphal growth, even without  
20 physical contact (Bécard, G. and Fortin, J. A., New Phytol. 108:211-218 (1988); Mosse, B. and Hepper, C. M., Physiol. Plant Pathol. 5:215-223 (1975); and Mosse, B., Can. J. Bot. 66:2533-2540 (1988)). However, the effects of either root exudates or extracts on spore germination or hyphal growth  
25 in vitro are very inconsistent (Harley, J. L. and Smith, S.E., Mycorrhizal symbiosis. Academic Press, London. p.483 (1983); Siqueira, J. O., Cultura axenica e monoxenica dos fungos micorizicos vesiculo-arbusculares. In: Proc. II Reuniao bras. micorrizas, Sao Paulo, Sec. Meio Ambiente. p.44-70 (1987)). A recent study however, indicated that  
30 the quality rather than the quantity of root exudates is an important factor for VAM fungal growth in vitro (Elias, K.S. and Safir, G. R., Appl. Environ. Microbiol. 53:1928-1933 (1987)). The authors suggested the presence  
35 of a transient VAM growth factor in the root exudates of phosphorus deprived clover plants.



Non-VAM species such as Brassica appear not to be colonized because they lack a diffusible growth stimulant present near the roots of compatible hosts (Glenn, M. G., et al., New Phytol. 110:217-225 (1988)).

- 5 This agrees with the suggestion that chemical signaling must take place in the early events of VAM establishment (Elias, K. S., and Safir, G. R., Appl. Environ. Microbiol. 53:1928-1933 (1987); Bécard, G. and Fortin, J. A., New Phytol. 108:211-218 (1988); Bonfante-Fasolo in Scannerini et al. (Scannerini, S., Smith, D., Bonfante-Fasolo, P. in  
10 Gianinazzi-Pearson, V. eds., Cell to cell signals in plant, animal and microbial symbiosis. Spring Verlag, Berlin. p.414 (1988)).

- Low molecular weight phenolic compounds are  
15 known to play important roles in a wide variety of plant-microbe systems. In the plant-Rhizobium symbiosis, flavonoids present in root exudates act in a regulatory fashion as inducers or repressors of nod genes on the symbiotic plasmids of the bacteria (Rolfe and Gresshoff,  
20 Ann. Rev. Pl. Physiol. Pl. Mol. Biol. 39:297-319 (1988). For instance, luteolin, which induces nod ABC gene expression in R. melliloti, may occur in low concentrations in the alfalfa rhizosphere and limit nodulation and nitrogen fixation (Kapulnik, Y, et al., Plant Physiol.  
25 84:1193-1196 (1987)). Flavonoids may also induce haustorial formation in parasitic plants (Steffens, J. C., et al. Ann. Bot. 50:1-7 (1982)); control plant microbial invasion (Bailey, J. A., ed. biology and molecular biology of plant-pathogen interactions. Spring Verlag, Berlin.  
30 p.415 (1986); Palacios, R. and Verma, D.P.S., ed. Molecular genetics of plant-microbe interactions. APS, St. Paul. p.401 (1988); Templeton, M.D. and Lamb, D. J., Plant, Cell Environ. 11:395-401 (1988); VanEtten, H.D., Phytochemistry 15:655-659 (1976)); act as natural auxin regulators  
35 (Jacobs, M. and Rubery, P.H., Science 241:346-349 (1987)) and are known to accumulate as a response to pathogen invasion (Templeton, M.D. and Lamb, C.J., Plant, Cell

Environ. 11:395-401 (1988)). Other low molecular weight phenolic compounds can induce gene expression of cytokinin biosynthesis and vir genes in pathogenic Agrobacterium (Conn, E. E., ed. Opportunities for phytochemistry in plant biotechnology. Plenum Press, New York, p.210 (1988));  
5 Powell, G. K., et al., Mol. Plant-Microbe Interactions. 1:235-242 (1988)); regulate cyclic processes in lichens. (Scannerini, S., et al., Cell to cell signals in plant, animal and microbial symbiosis. Spring Verlag, Berlin.  
10 p.414 (1988)). They can also act as allelopathic compounds and affect both VAM hyphal growth in vitro and root colonization (Wacker, T. L., et al., J. Chem. Ecol. (in press) (1989)).

The VAM fungi are obligate biotrophs. They may  
15 have lost the genetic capability for saprophytic growth (or have a depressed part of the required genome) during their long co-evolution with plants (Siqueira, J. O., Cultura axenica e monoxenica dos fungos micorizicos vesiculo-arbusculares. In: Proc. II Reuniao bras.  
20 micorizas, Sao Paulo, Sec. Meio Ambiente. p. 44-70 (1987)), thus permitting the host plant to have complete control of the fungal life cycle through interference with the replication of fungal nuclear DNA as recently suggested (Burggraaf, A.J.P. and Beringer, J.E., New Phytol.  
25 111:25-33 (1989)). In fact, the germination process is readily triggered after spore inhibition (Siqueira et al., Can. J. Microbiol. 31:965-972 (1985)), but at least for one species, nuclear DNA synthesis has not been found during in vitro development (Burggraaf, A.J.P. and Beringer, J.E.,  
30 New Phytol. 111:25-33 (1989)). Additionally, neither appresoria nor arbuscules (haustorium-like structures) have reported to form in the absence of living roots. It seems that both continued hyphal growth and differentiation are under host control. As observed for other obligate  
35 biotrophic fungi (Hoch, H.C. and Staples, R.C., Ann. Rev. Phytopathol. 25:231-247 (1987)) plant messengers or



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inducers may be needed as triggers for the invaders early growth, development and differentiation.

#### OBJECTS

It is therefore an object of the present invention to provide a method for producing plant infective VAM fungi in culture or in soil or other planting material. It is further an object of the present invention to provide novel compositions including VAM fungi. Further still, it is an object of the present invention to provide a method and compositions for stimulating plant growth or plant cell growth in the presence of VAM fungi. Further still, it is an object of the present invention to provide a method for improving plant productivity in soils that have phytotoxic levels of herbicides and other pesticides by stimulating the infective VAM fungi. Further still, it is an object of the present invention to provide a method which is simple and economical to use and is formulated in inexpensive agricultural compositions. These and other objects will become increasingly apparent by reference to the following description and the drawings.

#### IN THE DRAWINGS

Figure 1 shows the treatment of clover with isoflavonoids Clover A (formononetin, a natural form from the root extract); formononetin (synthetic); biochanin A compared with flavonoid compounds which are ineffective. Large increases in VAM fungus root colonization and clover growth are shown for formononetin, biochanin A and Clover A.

Figure 2 shows the growth of a VAM fungus having the characteristics of Glomus fasciculatum on nutrient media containing biochanin A; formononetin; and a control lacking either of these two chemicals. Large increases in fungus growth are caused by addition of formononetin or biochanin A in relation to the control.

Figures 3 to 6 show the spectral data for natural and synthetic formononetin in CD<sub>3</sub>OD and DMSO.

Figure 7 shows the percent VAM fungus root colonization as a function of concentration of biochanin A and formononetin.

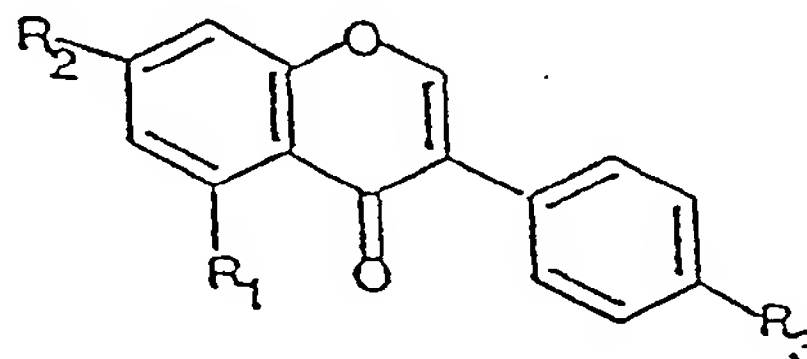
Figure 8 shows the increased leaf area of corn grown in unsterilized field soil in the presence of formononetin and biochanin A. The field soil contains the herbicide imazaquin at levels toxic to corn.

Figure 9 shows the reduction of herbicide injury to corn in the presence of added formononetin and biochanin A. The injury levels are for the corn plants described in Figure 8.

Figure 10 shows the increased dry weight of tops of the corn plants described in Figure 8 at four (4) weeks of age in the presence of formononetin and biochanin A.

#### 15 GENERAL DESCRIPTION

The present invention relates to an improvement in method for stimulating the growth of a plant material in the presence of vesicular-arbuscular mycorrhizal fungi which comprises: growing the plant material with the fungi in the presence of an isoflavonoid preferably of the formula



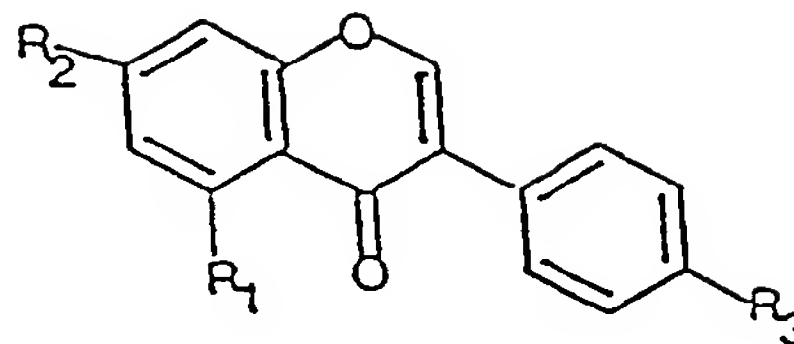
wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group consisting of hydrogen, hydroxy and alkoxy having 1 to 30 carbon atoms.

Further the present invention relates to a plant composition useful for stimulating the growth of a plant which comprises: an isoflavonoid compound preferably of the

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formula

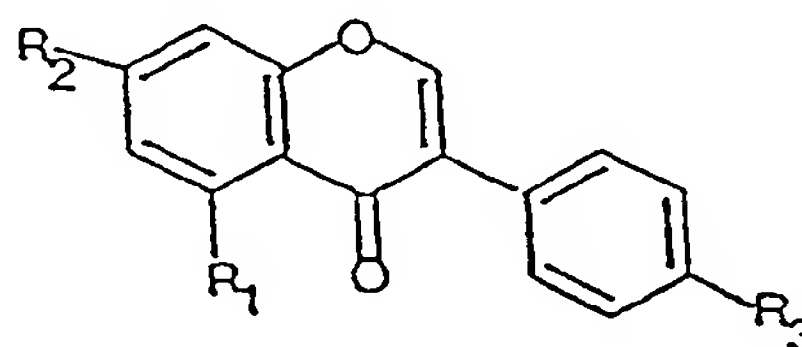
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wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxy and alkoxy having 1 to 30 carbon atoms; and a plant material containing the isoflavonoid compound as an additive in an amount which stimulate the growth of the plant material when the plant material is grown in the presence of VAM fungi.

Further still the present invention relates to a method for growing vesicular-arbuscular mycorrhizal fungi including spores of the fungi useful for stimulating plant growth which comprises: growing the vesicular-arbuscular mycorrhizal fungi in the presence of an amount of an isoflavonoid preferably of the formula

20



25

wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxyl and alkoxy containing 1 to 30 carbon atoms so that the fungi produced stimulate the growth of the plant.

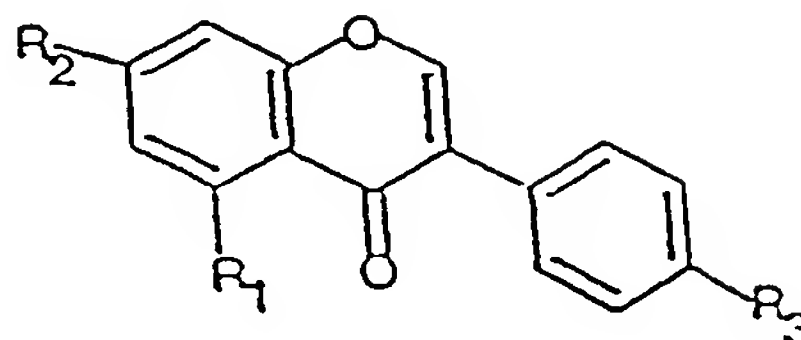
The present invention also relates to a fungal composition which comprises: vesicular-arbuscular mycorrhizal fungi which have been grown in the presence of

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an isoflavonoid preferably of the formula

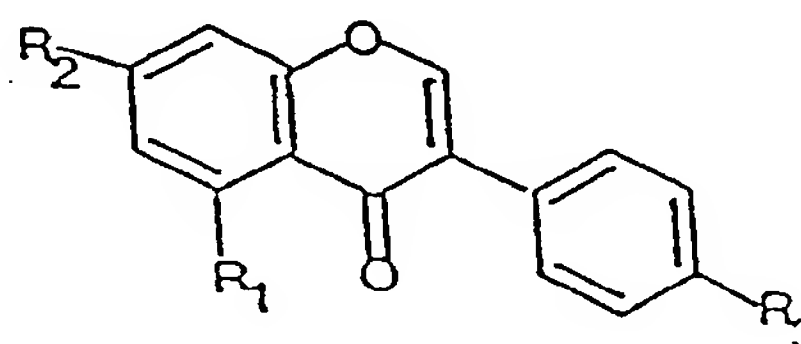
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wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxyl and alkoxy containing 1 to 30 carbon atoms.

Further, the present invention relates to a method for stimulating the growth of a plant in culture which comprises: growing a plant or cells of the plant in a culture solution containing vesicular-arbuscular mycorrhizal fungi and an isoflavonoid preferably of the formula:

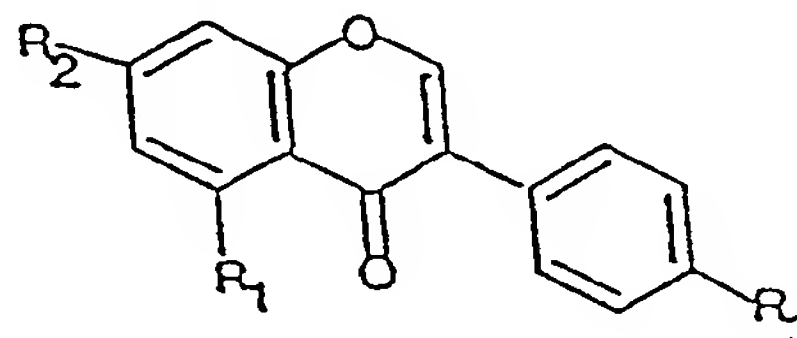
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wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxyl and alkoxy containing 1 to 30 carbon atoms.

Finally the present invention relates to a novel compound of the formula:

30

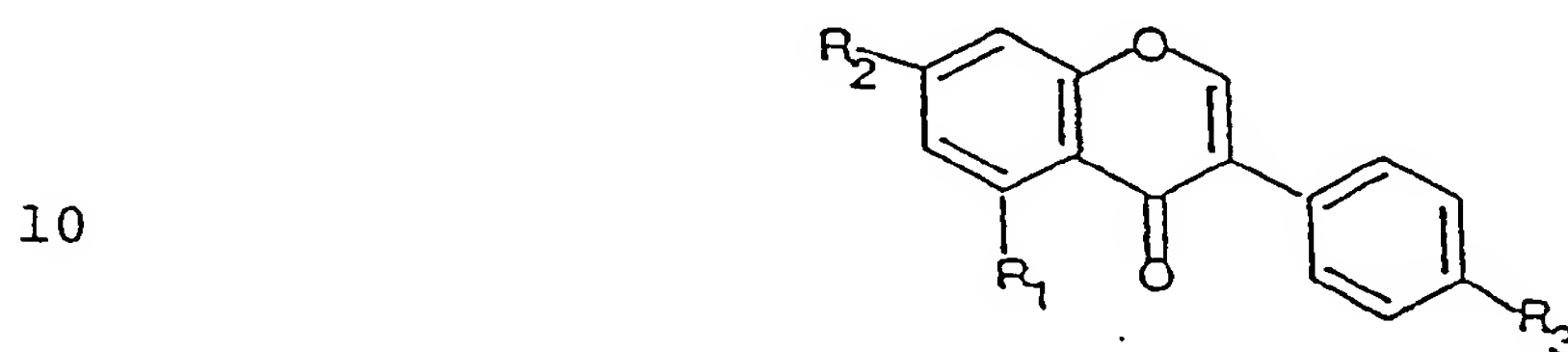


wherein  $R_1$ ,  $R_2$ , and  $R_3$  are selected from the group consisting of alkoxy groups containing 1 to 30 carbon atoms.

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Various long chain alkoxy groups can be attached to the isoflavonoid. These groups are selected so as not to interfere with the stimulation of the VAM fungi.

The most preferred isoflavonoid compounds of the present invention are of the formula:



wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are as shown in Table 1.

15

Table 1

	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
(1)	H	H	H
(2)	OH	OH	OMe
20 (3)	OMe	OMe	OMe
(4)	H	OH	OMe
(5)	OH	OMe	OMe
(6)	H	OMe	OMe
(7)	H	OMe	OH
25 (8)	H	H	OMe

Most preferred are biochanin A (2) and formononetin (4).

Isoflavonoid compounds that stimulate fungal hyphal growth in vitro were isolated from host roots and chemically identified as formononetin and biochanin A.

30 Formononetin was later synthesized. Thus, large VAM fungi growth stimulation was found in response to formononetin and biochanin A at 5 ppm concentrations. These root factors were used as a plant growth promoting substance, either by increasing VAM formation with indigenous soil  
35 fungal populations or by stimulating formation of the external mycelium network in the soil which in turn

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increases the plant's capability of taking up nutrients and water from the soil.

The plant material can be rooted plants or plant tissue cells, organs, seeds or other parts of the plant and can be grown in culture with the VAM fungi. The preferred plant materials are corn, soybean, sorghum, asparagus, leek, onion, Taxus sp., coffee, clover, citrus, sea oats, wheat, potatoes and other crop plants, particularly those plants having roots which are colonized by the VAM fungi. The isoflavonoid is used in an amount between about 0.1 and 400 ppm in soil or planting mixes. Planting mixes can include vermiculite, polystyrene beads, peat moss and other fillers and growth factors. In tissue culture, the isoflavonoid is present in an amount between about 0.0001 and 400 ppm with the plant material and VAM fungi.

The isoflavonoid can be applied to the soil or planting mix either before or after the plants are planted. Preferably the isoflavonoid is applied at the time of planting of the seed. The VAM fungi can also be applied or they can be naturally present in the soil.

The isoflavonoid can be applied to the plant material, e.g. either to the seed or a propagule. Preferably the isoflavonoid is coated on the seed using an adhesive such as methyl cellulose, which is compatible with plant growth. The isoflavonoid can also be impregnated into the seed. Preferably the VAM fungi and seeds coated with the isoflavonoid are applied together. The VAM fungi can also be cultured with the isoflavonoid.

The preferred VAM fungi are in the genus Glomus such as G. fasciculatum, G. intraradices and G. etunicatum.

These VAM fungi are particularly important commercially. It is preferred that the VAM fungi are grown in the presence of the isoflavonoid in an amount between about 0.0001 and 400 ppm in the culture medium. The culture medium contains sources of carbon, nitrogen,



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minerals and vitamins as is known to those skilled in the art.

The isoflavonoids can be applied in a liquid agricultural carrier with a dispersant which maintains the isoflavonoid in solution in an amount between about 0.1 and 400 micrograms per ml. Preferred dispersants are lower alkanols, particularly methanol, with various surfactants including anionic and cationic surfactants. Other organic solvents can be used to form emulsions of the isoflavonoid in water. The isoflavonoids can be provided in a solid mixture including the dispersant and the isoflavonoid. The composition can be formulated in solid carriers which aid in dispersing the isoflavonoid in the soil or planting material. The isoflavonoid is present in an amount between about 0.1 and 400 ppm by weight of the solid carrier.

The isoflavones can be formulated as wettable powders, flow concentrates, emulsifiable concentrates, granular formulations and the like.

Wettable powders can be prepared by grinding together about 20% to 45% by weight of a finely divided carrier such as kaolin, bentonite, diatomaceous earth, attapulgite, or the like, 45% to 80% by weight of the active compound, 2% to 5% by weight of a dispersing agent such as sodium lignosulfonate, and 2% to 5% by weight of a nonionic surfactant, such as octylphenoxy polyethoxy ethanol, nonylphenoxy polyethoxy ethanol or the like.

A typical flowable liquid can be prepared by admixing about 40% by weight of the active ingredient with about 2% by weight of a gelling agent such as bentonite, 3% by weight of a dispersing agent such as sodium lignosulfonate, 1% by weight of polyethylene glycol and 54% by weight of water.

A typical emulsifiable concentrate can be prepared by dissolving about 5% to 25% by weight of the active ingredient in about 65% to 90% by weight of N-methyl-pyrrolidone, isophorone, butyl cellosolve, methylacetate or the like and dispersing therein about 5%

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to 10% by weight of a nonionic surfactant such as an alkylphenoxy polyethoxy alcohol. This concentrate is dispersed in water for application as a liquid spray.

When the isoflavonoids are used for soil  
5 treatment, the compounds may be prepared and applied as granular products. Preparation of the granular product can be achieved by dissolving the active compound in a solvent such as methylene chloride, N-methylpyrrolidone or the like and spraying the thus prepared solution on a granular  
10 carrier such as corncob grits, sand, attapulgate, kaolin or the like.

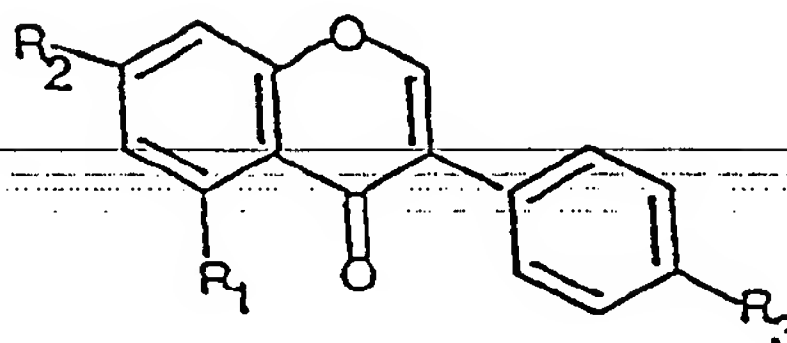
The granular product thus prepared generally comprises about 3% to 20% by weight of the active ingredient and about 97% to 80% by weight of the granular  
15 carrier.

The isoflavonoids can also be mixed with a herbicide or pesticide which is applied to the plants or applied before or after the application of the herbicide or pesticide. The VAM fungi function as a "safener" in the  
20 presence of the isoflavonoids, and overcome injury caused by the herbicides or pesticides. Injury caused by imidazolinone herbicides, such as imazaquin and imazethapyr, and pendimethalin are particularly overcome by the method of the present invention. Best results are seen  
25 when the composition is applied the year following a herbicide application to fields showing residual levels of herbicide sufficient to cause injury to crops planted into the field.

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The novel compound of the formula



10 wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group  
consisting of alkoxy groups containing 1 to 30 carbon atoms  
has increased water solubility over the known hydroxy  
substituted isoflavonoids. In the preparation of the novel  
15 compounds a hydroxy flavonoid such as (2) in Table 1 is  
alkylated by reaction with an alkyl halide (chloride,  
bromide or iodide) in the presence of a base or acid and  
organic solvents such as dichloromethane acetone, and the  
like. The mixture is refluxed for several hours,  
preferably 12 to 24 hours. Essentially the reaction is a  
20 conventional alkylation reaction of phenols.

The isoflavonoids in Table 1 are available  
commercially except for the peralkoxy isoflavonoids. They  
have been prepared by alkylation of the isoflavonoids in  
Table I.

25 SPECIFIC DESCRIPTION

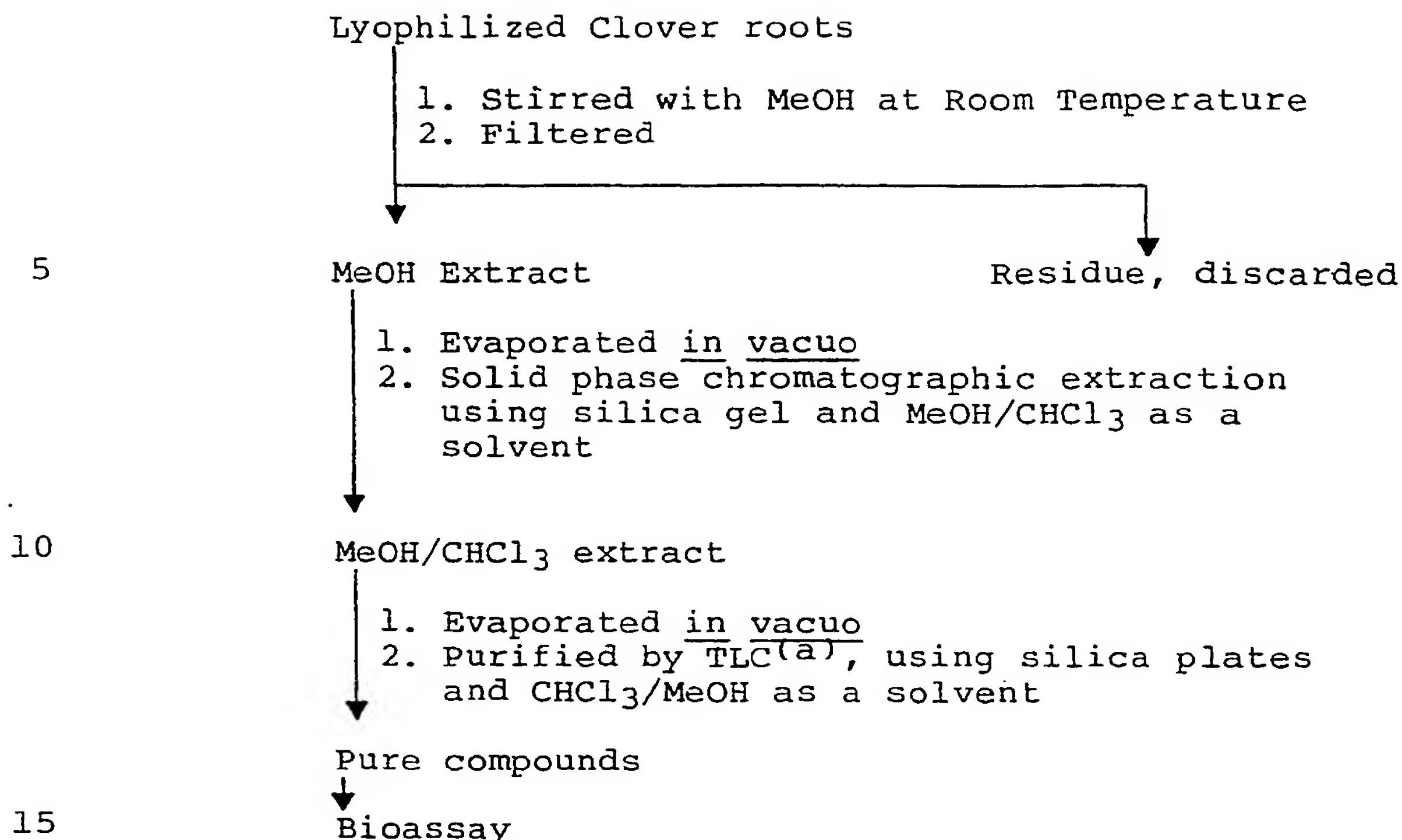
Example 1

The isoflavonoids were initially isolated from  
clover roots and then were identified as formononetin and  
biochanin A. The isoflavonoids were isolated from clover  
30 roots by the general scheme shown in Table 2. Figures 3 to  
6 show the spectral data for natural and synthetic  
formononetin.

35

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Table 2



(a) TLC is thin layer chromatography.

Two active compounds from TLC were chemically characterized. The high R<sub>f</sub> compound, clover A, was found to be identical to formononetin, 7-hydroxy, 4'-methoxy isoflavone. This was confirmed by <sup>1</sup>H, <sup>13</sup>C-NMR, long range COSY and a direct comparison with synthetic Formononetin as in Figures 3 to 6.

The lower R<sub>f</sub> compound was eluted with CHCl<sub>3</sub>/MeOH and afforded a pale brown solid, H-NMR (CDCl<sub>3</sub>/DMSO) δ 8.00 (1H, s, H-2), 7.40 (2H, d, J=8Hz, H-2', H-6'), 6.90 (2H, d, J=8Hz, H-3', H-5'), 6.35 (1H, d, J=1.8Hz, H-8), 6.25 (1H, d, J=1.8Hz, H-6), 3.75 (3H, s, OMe); Molecular ion at m/z 284. Comparison of these data to the reported values of biochanin A confirmed that the low R<sub>f</sub> compound isolated from clover root is also biochanin A.

The two compounds chemically identified were 7-hydroxy, 4'-methoxy isoflavone (Table 1, 4). (formononetin) and 5,7-dihydroxy, 4'-methoxy isoflavone (Table 1, 2)

-17-

(biochanin A). These compounds were active in the 0.1 to 400 ppm concentration range and are unknown for this use.

Initially, root exudates from Ladino white clover seedlings were obtained from plants at 2 weeks of age that had been grown in a nutrient solution with no phosphorus as shown in Table 3.

Table 3

Composition of nutrient solution

	<u>Components</u>	<u>Concentration, mg/l</u>
10	KNO <sub>3</sub>	606.60
	Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	656.40
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	240.80
	H <sub>3</sub> BO <sub>3</sub>	2.86
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.81
15	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.22
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.08
	H <sub>2</sub> MoO <sub>4</sub>	0.02
	Fe-tartrate	5.00

20 Adjust to pH 6.8 with 0.1N NaOH

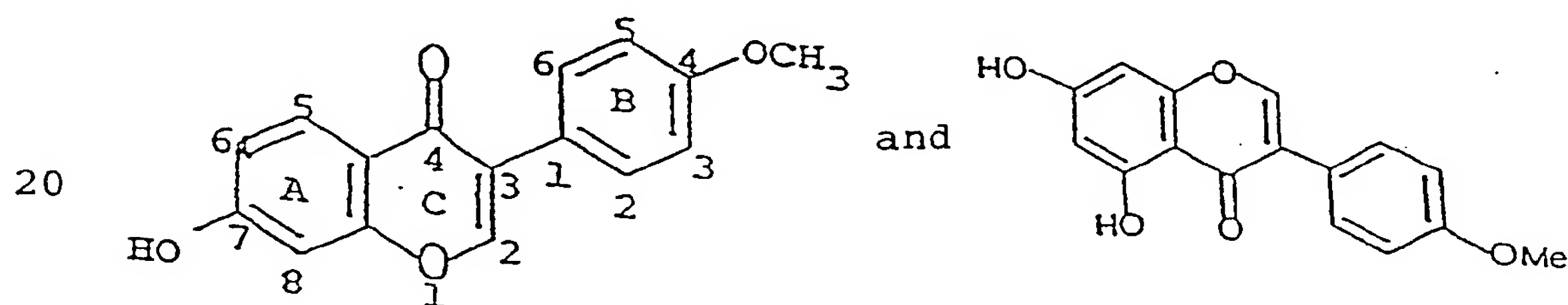
The root exudates of these seedlings were lyophilized and extracted with MeOH at room temperature. The MeOH extract was dried in vacuo and the resultant crude extract was chromatographed on silica gel thin layer plates using a 4:1

25 chloroform-methanol solvent system. Three bands, detected under long and short wavelength UV light (254 and 366 nm), were eluted separately using 1:1 MeOH-CHCl<sub>3</sub> and evaporated to dryness. These pure fractions were bioassayed for their ability to stimulate VAM hyphal growth on agar media and

30 found to be active in this capacity. Since the exudate fraction had low concentrations of active compound, clover roots were extracted in order to obtain these compounds. The clover roots were obtained from plants less than 2 weeks of age that had been grown in unsterilized sand and

35 watered with distilled water daily. The roots were lyophilized at 5°C and extracted with MeOH at room temperature. The MeOH solvent was removed under vacuum at

40°C and the resultant extract was partially purified by solid phase extraction on silica gel using MeOH under vacuum, evaporated to dryness using a rotary evaporator and the resultant product purified on tapered thin layer plates (silica, 4:1 chloroform-methanol). Three bands with identical R<sub>f</sub> values to the root exudate were collected, extracted, and processed as before. The pure compounds obtained were identical to the compounds from the root exudate in VAM biological activity. Two compounds were chosen, the highest and middle R<sub>f</sub> value compounds, for immediate identification and for VAM fungal growth studies. These compounds were characterized by <sup>1</sup>H- and <sup>13</sup>C-NMR and MS methods and found to be isoflavones with one methoxy substituent attached to ring B and hydroxy group(s) on ring A as follows:



H-NMR spectral analysis showed the active compound to be identical to 7-hydroxy-4'-methoxyisoflavone (formononetin compound 4 Table 1) and 5,7-dihydroxy-4'-methoxy isoflavone, (Biochanin A, Compound 2, Table 1).

#### (1) Crude Production of Isoflavonoids

White clover (Trifolium repens cv. Ladino) seeds and seeds from other plant types were surface sterilized with 70% ethyl alcohol for 30 seconds followed by 0.1% HgCl<sub>2</sub> in 1 mM HCl for 5-7 minutes followed by exhaustive washing with sterile distilled water.

The seeds were then placed on moist filter paper in a sterile petri dish incubated at 23°C in the dark for 2 days to allow germination. Fifty seedlings were transferred from the petri dish to moist cheese cloth in a



sterilized glass dish containing 100 ml of sterilized Hoagland's nutrient solution, without phosphorus. The dishes were enclosed in sterile clear plastic bags, tightly sealed and the seedlings grown under 16 hour day length  
5 fluorescent lights (4.5 lux). The Hoagland's solution was replaced at weekly intervals.

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At weekly intervals, exudates or extracts were collected from the roots of these clover and other seedlings. Weekly, solutions are pooled, filter  
10 sterilized, rotary evaporated to 1/10 original volume, filter sterilized again, lyophilized, and stored at 4°C.

## (2) Chemical Characterization

The chemical characterization of active fractions containing the isoflavonoids from various plant  
15 sources was according to established procedures. At each stage of purification or characterization, portions of extract were produced for determination of VAM fungal growth stimulating activity. The results of the bioassay enabled further chemical characterization of the  
20 isoflavonoids.

### Example 2

Based on the chemistry of the compounds formononetin and biochanin A isolated from clover roots, the VAM biological activity of additional commercially  
25 available flavonoids were investigated along with formononetin and biochanin A and the experiments were conducted to determine if other compounds would stimulate VAM fungal root colonization and subsequent plant growth. Clover plants were used and grown in a substrate infested  
30 with either Glomus intraradices or a fungus resembling Glomus fasciculatum.

Disinfected pre-germinated clover seeds were transplanted into a sand soil mix (2:1) in plastic inserts containing four 60 cc individual cells. Each four-cell  
35 unit was placed in 90 x 15 mm plastic petri dish bottom to avoid chemical contamination and to facilitate watering. Free VAM propagule sand soil mix was infested with a VAM

5 fungal soil inoculum of either a Glomus fasciculatum-like fungus or Glomus intraradices. The substrate had a neutral pH, low exchange capacity, and a low to moderate fertility level. The VAM soil inoculum was obtained from pot culture kept in the greenhouse for at least 4 months. Dried inoculum was mixed thoroughly with the substrate in order to achieve a final spore density ranging from 2 to 5 spores per gram of the soil mix.

10 Before transplanting the seeds, between 5 to 10 ml of 5 ppm solutions of either Clover A (isolated natural form of formononetin) or other pure chemicals were delivered per each cell. The inserts were placed in plastic trays and transferred to a full light growth chamber set at 14 hours of light per day plus 10 hours of  
15 dark per day length.

The plants were watered twice a day with distilled water and allowed to grow for 4 weeks, after which they were harvested. Growth responses were measured as shoot fresh weight. Roots were separated from the soil  
20 and used for VAM root colonization assessments.

As shown in Figure 1, the isoflavonoids formononetin (both the natural and synthesized form) and biochanin A stimulated VAM fungal root colonization as well as clover plant growth when the plants were allowed to grow  
25 in a VAM fungus infested substrate. The other ineffective compounds tested, genestein, 7-8 dihydroxyflavone, chrysin, luteolin, naringenin and hesepertin failed to improve plant growth or VAM fungus root colonization. Figure 7 shows the affect of concentration of biochanin A and formononetin on  
30 percent VAM fungus colonization for clover. Similar increases in growth and fungal root colonization have been observed for corn and sorghum after treatment with formononetin and biochanin A.

### Example 3

35 Figures 8 and 10 show the growth and Figure 9 shows the herbicide injury level of corn seedlings grown in an unsterilized field soil in the presence or absence of

-21-

formononetin (Form), biochanin A (Bio) or the VAM fungus Glomus intraradices (Myc) at an added rate of 7 spores/gram soil. The field soil contained an initial residual level of 13 ppb of the herbicide imazaquin and an indigenous VAM fungus level of approximately 7 spores/gram soil. Leaf area measurements of the third leaf from the soil were taken at 2 and 3 weeks after the pre-germinated corn seeds were transplanted and grown in the field soil in 3 1/2" x 6" Styrofoam pots in a growth chamber. The growth chamber had a day temperature of 30°C and a night temperature of 25°C and 14 hours of light per day plus 10 hours of dark per day. Figure 8 shows the increased areas of corn leaves at 2 and 3 weeks after treatment with formononetin (-MycForm), biochanin A (-MycBio), or the VAM fungus Glomus intraradices in comparison to the control plants (-myc control). Adding biochanin A or formononetin along with the added VAM fungus inoculum (+MycBio or +MycForm) also increased corn leaf areas.

Figure 9 shows the herbicide injury of the corn seedlings described in Figure 8 (above) at 2 and 3 weeks after transplanting. The injury rating scale based on the intensity of leaf chlorosis ranged from 0=no injury to 5=severe. Both formononetin and biochanin A reduced the herbicide injury of the corn plants in comparison to plants not receiving these compounds.

Figure 10 shows the percent increase in dry weight of tops of the corn plants described in Figure 8 in comparison to non VAM inoculated control at four weeks of age. Treatment with formononetin or biochanin A increased the dry weight of tops of the corn plants.

#### Example 4

##### 1. Spore Production

VAM spores of several fungal species were obtained by growing sorghum plants in pots with disinfected soil in the presence of spores to be multiplied. These spores germinate and infect the growing plants with the resulting mycorrhizal infections producing new spores in

the soil in 3 to 4 months. The resulting spores were used for experimental purposes. After sporulation, pot cultures were stored at 4°C for at least 30 days before use. This VAM infested soil was wet sieved to eliminate most of the soil and organic debris which was collected on the sieve. Next a modified centrifugation-flotation technique in ~~Ficoll solutions of various densities was used to isolate~~ spores from the soil debris. Organic debris was further removed from the spore suspension by hand with a Pasteur pipet under a dissecting microscope. Spores were then surface-sterilized with a solution containing 2% Chloramine-T (w/v), +200 ppm of Streptomycin, before use for germination and hyphal growth studies.

## 2. Germination and Hyphal Growth Bioassay

Formononetin and biochanin A were added to agar at a range of concentrations per volume of media. Germination and hyphal elongation of the VAM spores was monitored. Germination was defined as a germ tube that was at least twice the diameter of the spore. Hyphal elongation effects were the mean hyphal length of only those spores that germinate. The bioassay time was shortened to 2 weeks from 4 weeks. There were two additional methods for bioassaying fungal growth. The first method used liquid culture, and the second involved a membrane sandwich containing VAM spores superimposed over sterile sand containing nutrients. Both of these procedures produced similar results in 1 week. The results from agar media are shown in Figure 2 for a VAM fungus resembling Glomus fasciculatum.

### Example 5

#### Methylation

Formononetin (250 mg) and biochanin A (500 mg) were separately dissolved in acetone (25 ml) and stirred with anhydrous K<sub>2</sub>CO<sub>3</sub> (30 g), (5 min) at room temperature. Dimethyl sulphate (3 ml each) was added to the above reaction mixture and refluxed for 18 hours. Reaction mixture was cooled, filtered separately and rotary

-23-

evaporated with methanol. The crude products were crystallized from methanol and dried under vacuum. Melting points were taken for the methylated product on a Kofler hot stage apparatus and were uncorrected.

- 5 The physical properties of the permethoxy isoflavonoids are as follows:

MP, 7-methoxy, 4'-methyl isoflavone=155-156°C

(white, plate-like crystals),  
Molecular weight 282.

10

MP, 5,7-dimethoxy, 4'-methoxy

isoflavone=158-160°C

(pale yellow, plate-like  
crystals), Molecular weight 312.

Solubility: 5,7-dimethoxy, 4'-methoxy isoflavone  
and 7-methoxy, 4'-methyl  
isoflavone = soluble in methanol,  
methanol-water, partially  
soluble in CHCl<sub>3</sub>.

15

- 20 The increased water solubility of these isoflavonoids makes them particularly useful.

#### Example 6

- 25 The method of Example 4 can be used to methylate other hydroxy containing isoflavonoids shown in Table 2, such as formononetin. This process produces known compounds.

It is intended that the foregoing description be only illustrative of the present invention and that the present invention be limited only by the hereinafter appended claims.

30

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WE CLAIM:

-1-

In a method for stimulating the growth of a plant material in the presence of vesicular-arbuscular

mycorrhizal fungi, the improvement which comprises:  
growing the plant material with the fungi in the  
5 presence of an isoflavonoid.

-2-

A plant material composition useful for stimulating the growth of a plant which comprises:

- (a) an isoflavonoid compound; and
- (b) a plant material containing the compound  
5 as an additive in an amount which stimulates the growth of the plant material to a plant when the plant material is grown in the presence of VAM fungi.

-3-

A method for growing vesicular-arbuscular mycorrhizal fungi including spores of the fungi useful for stimulating plant growth which comprises:

- growing the vesicular-arbuscular mycorrhizal  
5 fungi in the presence of an amount of an isoflavonoid so that the fungi produced are stimulating to the growth of the plant.

-4-

An agricultural composition useful for stimulating the growth of plant material in the presence of vesicular-arbuscular mycorrhizal fungi: and

- (b) an agricultural carrier, wherein the  
5 isoflavonoid is present in an amount between 0.1 and 400 parts by weight of the carrier wherein the composition stimulates the growth of the plant material in the presence of the fungi.



-25-

-5-

A fungal composition which comprises:  
vesicular-arbuscular mycorrhizal fungi which  
have been grown in the presence of an isoflavonoid.

-6-

A fungal composition which comprises in  
admixture:

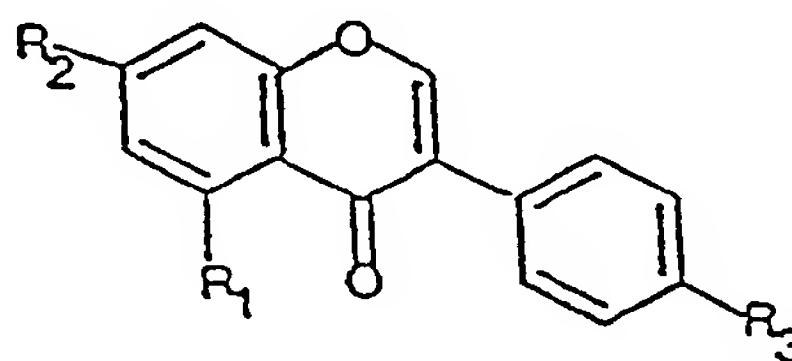
- (a) an isoflavonoid; and
  - (b) a vesicular-arbuscular mycorrhizal fungi
- 5 which is stimulated by the isoflavonoid.

-7-

A method for alleviating pesticide or herbicide  
injury in a soil in the presence of vesicular-arbuscular  
mycorrhizal fungi where pesticides or herbicides have been  
applied to the soil and are present at levels toxic to  
5 plants and growing the plant material with the fungi in the  
presence of an isoflavonoid.

-8-

In a method for stimulating the growth of a  
plant material in the presence of vesicular-arbuscular  
mycorrhizal fungi, the improvement which comprises:  
growing the plant material with the fungi in the  
5 presence of an isoflavonoid of the formula



10 wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group  
consisting of hydrogen, hydroxy and alkoxy having 1 to 30  
carbon atoms.

-26-

-9-

In a method for stimulating the growth of a plant material in the presence of vesicular-arbuscular mycorrhizal fungi, the improvement which comprises:

~~growing the plant material with the fungi in the~~  
5 ~~presence of an amount of an isoflavonoid compound selected~~  
~~from the group consisting of formononetin and biochanin A~~  
~~and mixtures thereof which stimulates the growth of the~~  
~~plant material.~~

-10-

A method for stimulating the growth of a plant planted in soil or planting mixes containing vesicular-arbuscular mycorrhizal fungi which comprises:

5 applying an amount of an isoflavonoid compound selected from the group consisting of formononetin and biochanin A and mixtures thereof to the soil so as to stimulate the growth of the plant.

-11-

The method of Claim 10 wherein the amount of the isoflavonoid is applied in an amount between about 0.1 and 400 ppm in the soil or planting mixes.

-12-

The method of Claim 11 wherein the fungi are applied with the isoflavonoid.

-13-

The method of Claim 10 wherein the plant is selected from the group consisting of plants having roots colonized by these fungi.

-27-

-14-

The method of Claim 10 wherein a seed or a propagule is planted and the isoflavonoid is applied at about the time of planting.

-15-

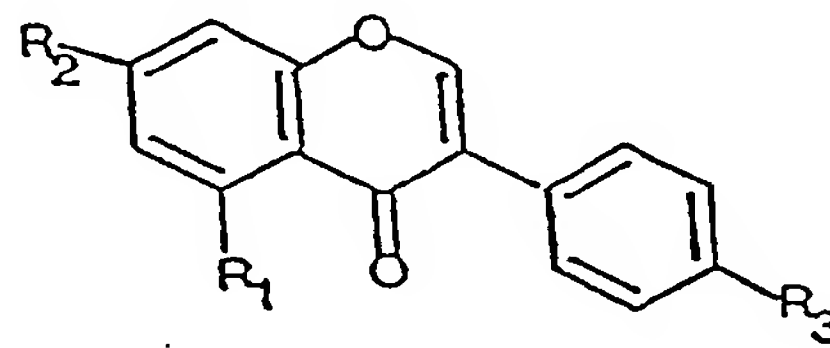
The method of Claim 14 wherein the seed or a propagule is coated with the isoflavonoid.

-16-

A plant material composition useful for stimulating the growth of a plant which comprises:

(a) an isoflavonoid compound of the formula

5



10 wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxy and alkoxy having 1 to 30 carbon atoms;

15 (b) a plant material containing the compound as an additive in an amount which stimulates the growth of the plant material to a plant when the plant material is grown in the presence of VAM fungi.

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A plant material composition useful for stimulating the growth of a plant from a seed or propagule of the seed when planted which comprises:

5 (a) a isoflavonoid compound selected from the group consisting of formononetin and biochanin A and mixtures thereof; and

(b) a seed or propagule containing the compound as an additive in an amount which stimulates the growth of the seed or propagule when planted.

-28-

-18-

The composition of Claim 17 wherein the seed or propagule is selected from the group consisting of all plants colonized by vesicular-arbuscular mycorrhizal fungi.

-19-

The composition of Claim 15 wherein vesicular-arbuscular fungi are admixed with the seed or propagule.

-20-

The composition of Claim 17 wherein the isoflavonoid is coated on the seed or propagule.

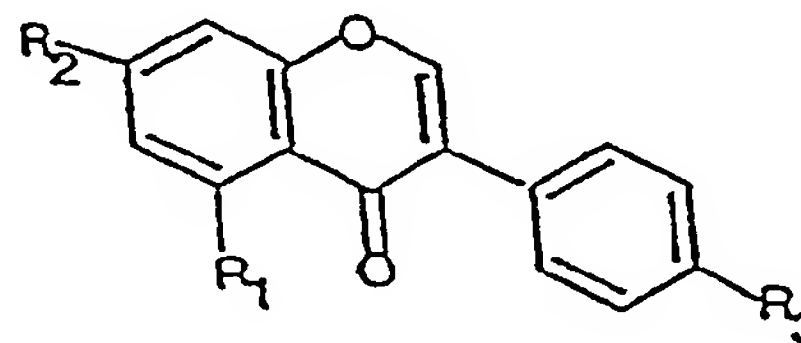
-21-

The composition of Claim 17 wherein the isoflavonoid is impregnated into the seed or propagule.

-22-

A method for growing vesicular-arbuscular mycorrhizal fungi including spores of the fungi useful for stimulating plant growth which comprises:

growing the vesicular-arbuscular mycorrhizal  
5 fungi in the presence of an amount of an isoflavonoid of  
the formula



10

wherein  $R_1$ ,  $R_2$  and  $R_3$  are selected from the group consisting of hydrogen, hydroxyl and alkoxy containing 1 to 30 carbon atoms so that the fungi produced are stimulating  
15 to the growth of the plant.

-29-

-23-

A method for growing, which includes fungal sporulation, vesicular-arbuscular mycorrhizal fungi useful for stimulating plant growth which comprises growing the vesicular-arbuscular fungi in the presence of an amount of an isoflavonoid compound selected from the group consisting

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5 of formononetin and biochanin A and mixtures thereof so that the fungi produced stimulate the plant growth.

-24-

The method of Claim 23 wherein the fungi are selected from the group consisting of fungi which colonize roots of the plant.

-25-

The method of Claim 23 wherein an amount of the compound between about 0.1 and 400 ppm is provided in a medium for growing the fungi.

-26-

The method of Claim 25 wherein the medium contains a source of carbon, nitrogen and vitamins and minerals which stimulate the growth of the fungi.

-27-

The method of Claim 25 wherein the medium contains plant materials which stimulate growth of these fungi.

-28-

The method of Claim 25 wherein the medium contains a source of carbon, nitrogen and vitamins and minerals which stimulate the growth of the fungi and wherein the medium contains plant materials which stimulate

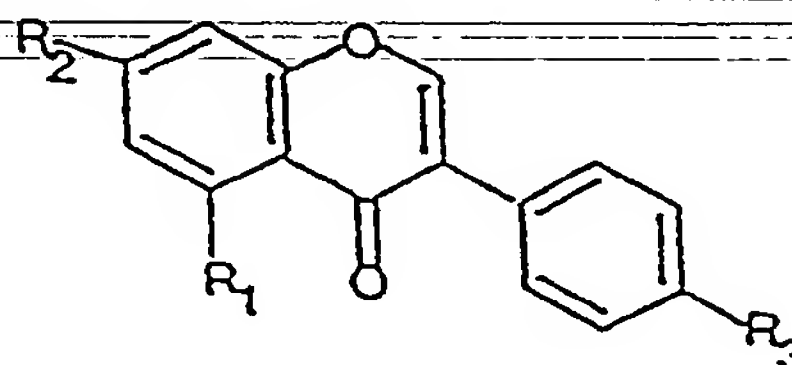
5 growth of the fungi.

-30-

-29-

An agricultural composition useful for stimulating the growth of plant material in the presence of vesicular-arbuscular mycorrhizal fungi:

- (a) an essentially pure isoflavonoid compound of  
5 the formula:



10

wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group consisting of hydrogen, hydroxyl and alkoxy containing 1 to 30 carbon atoms; and

- (b) an agricultural carrier, wherein the  
15 isoflavonoid is present in an amount between 0.5 and 400 parts by weight of the carrier wherein the composition stimulates the growth of the plant material in the presence of the fungi.

-30-

An agricultural composition useful for stimulating the growth of a plant or a seed of the plant in the presence of vesicular-arbuscular mycorrhizal fungi which comprises:

- 5 (a) an essentially pure isoflavonoid compound selected from the group consisting of chemically pure formononetin and biochanin A and mixtures thereof; and  
(b) an agricultural carrier, wherein the  
10 isoflavonoid is present in an amount between 0.1 and 400 parts by weight of the carrier, wherein the composition stimulates the growth of the plant or seed in the presence of the fungi.



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-31-

The composition of Claim 30 wherein the carrier contains nutrients which stimulate the growth of vesicular-arbuscular mycorrhizal fungi.

-32-

~~The composition of Claim 30 including in addition~~  
vesicular-arbuscular mycorrhizal fungi.

-33-

A method for stimulating the growth of a plant in culture which comprises:

- (a) providing a plant or cells of the plant in a culture solution containing vesicular-arbuscular mycorrhizal fungi and a compound selected from the group consisting of formononetin and biochanin A and mixtures thereof; and
- (b) growing the plant in the culture solution.

-34-

The method of Claim 33 wherein the plant is colonized by the fungi.

-35-

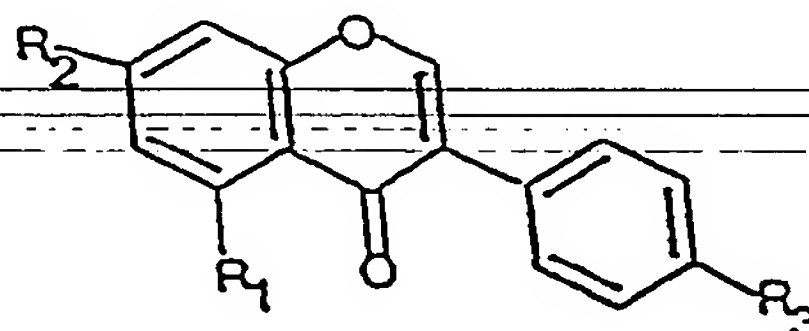
The method of Claim 33 wherein the amount of the flavonoid is between about 0.1 and 400 ppm in the solution.

-32-

-36-

A fungal composition which comprises:  
vesicular-arbuscular mycorrhizal fungi which have  
been grown in the presence of an isoflavonoid of the  
formula

5



10 wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group  
consisting of hydrogen, hydroxyl and alkoxy containing 1 to  
30 carbon atoms.

-37-

The composition of Claim 36 wherein the  
isoflavonoid is selected from the group consisting of  
formononetin and biochanin A.

-38-

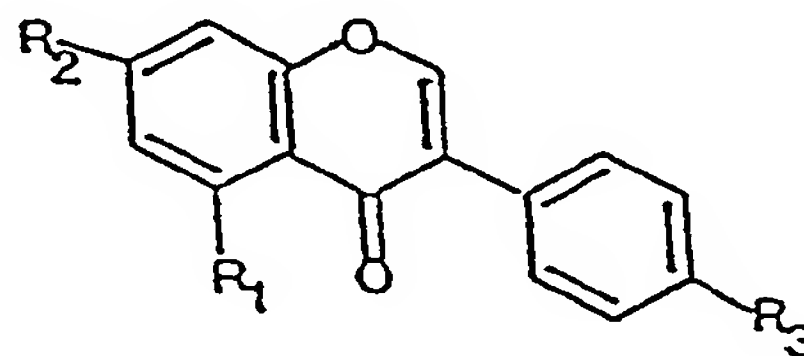
The composition of Claim 36 wherein the fungi are  
admixed with a plant material.

-39-

A fungal composition which comprises in  
admixture:

(a) an isoflavonoid of the formula:

5



10 wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group  
consisting of hydrogen, hydroxy and alkoxy containing 1 to  
30 carbon atoms; and

(b) a vesicular-arbuscular mycorrhizal fungi.

-33-

-40-

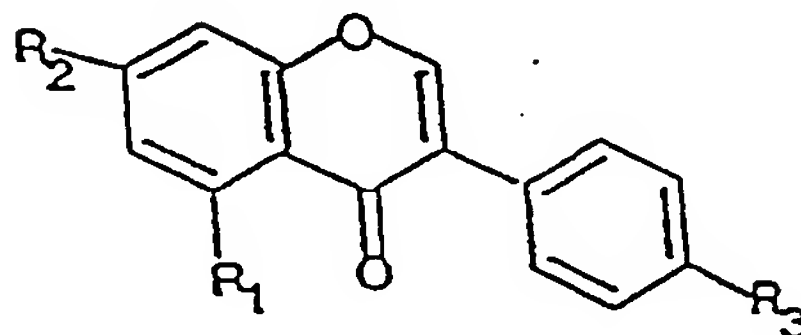
The composition of Claim 39 wherein the isoflavonoid is selected from the group consisting of formononetin and biochanin A.

-41-

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A compound of the formula:

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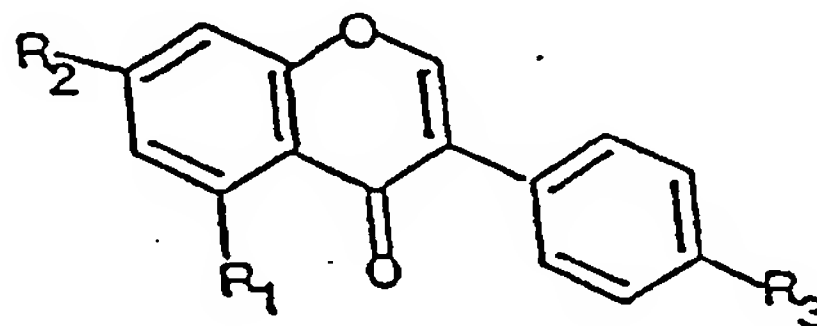
wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are selected from the group consisting of alkoxy groups containing 1 to 30 carbon atoms.

-42-

The compound of Claim 41 wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are methoxy.

-43-

A method for alleviating the injury to plants due to toxic levels of pesticides or herbicides in the soil by growing the plants in the presence of vesicular-arbuscular mycorrhizal fungi and an isoflavonoid of the formula:



wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> are selected from the group consisting of hydrogen, hydroxy and alkoxy containing 1 to 30 carbon atoms wherein there is enhanced growth of the plants in the soil.

-34-

-44-

The method of Claim 43 wherein the isoFlavonoids are selected from the group consisting of biochanin A and formononetin and mixtures thereof.

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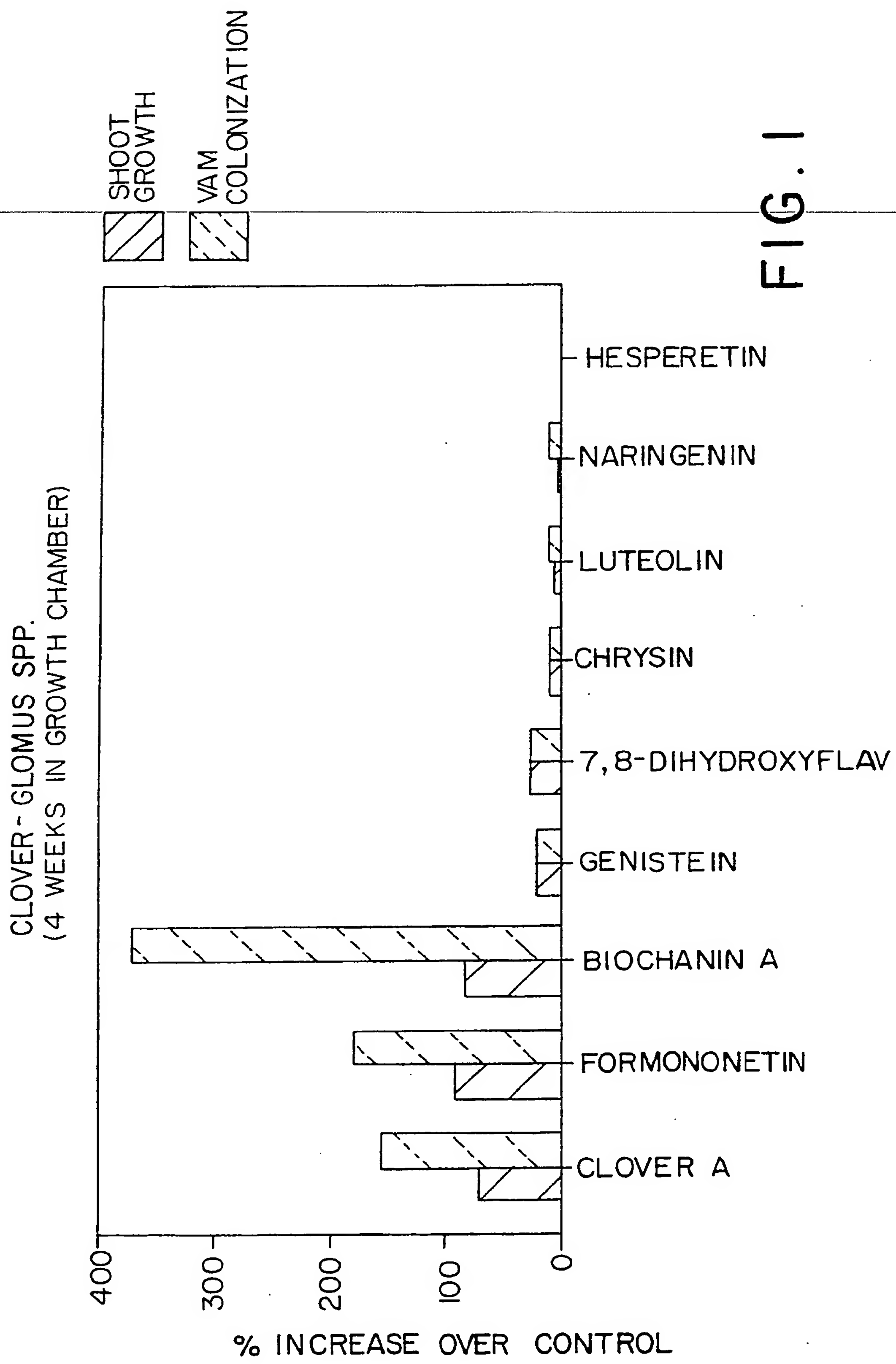
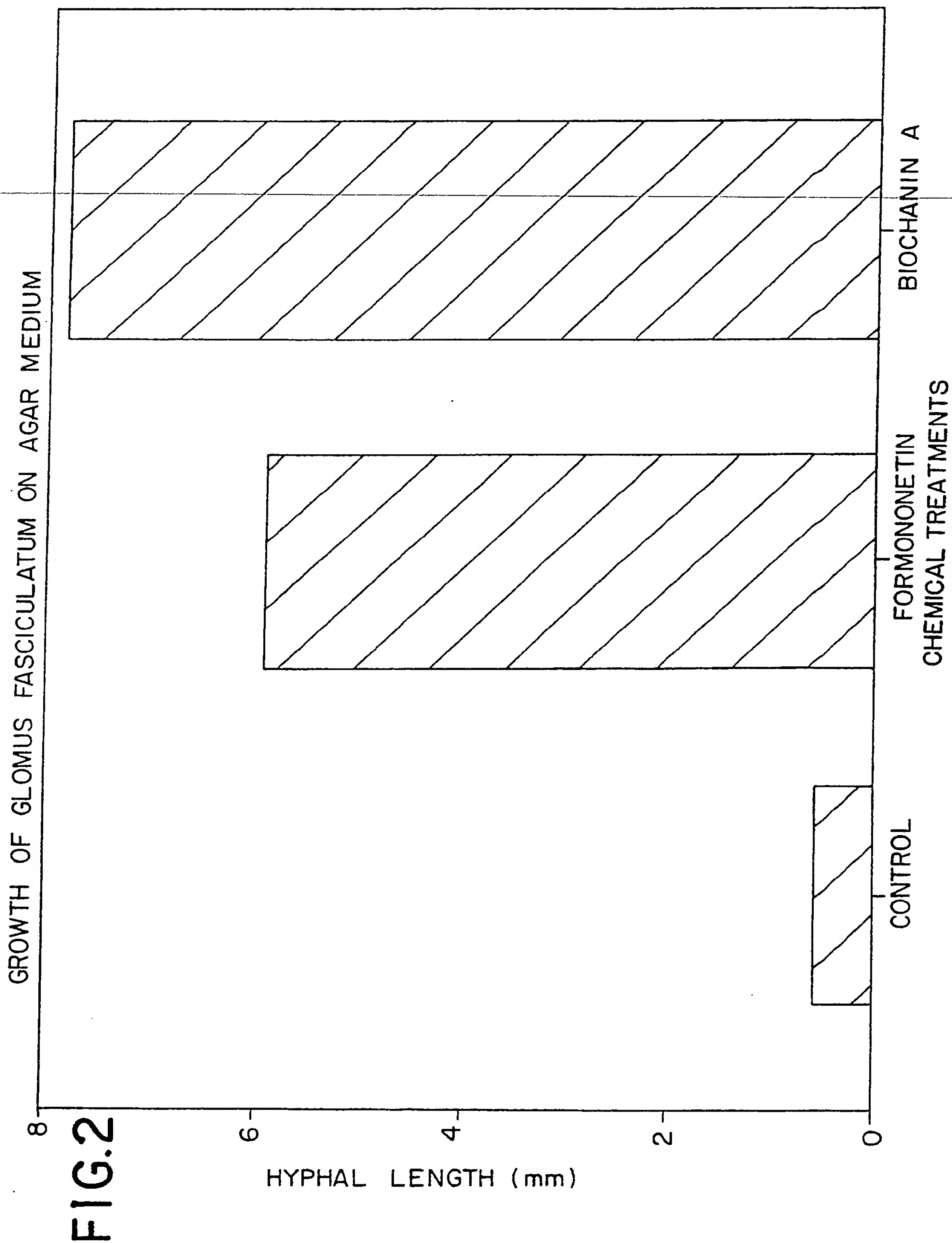
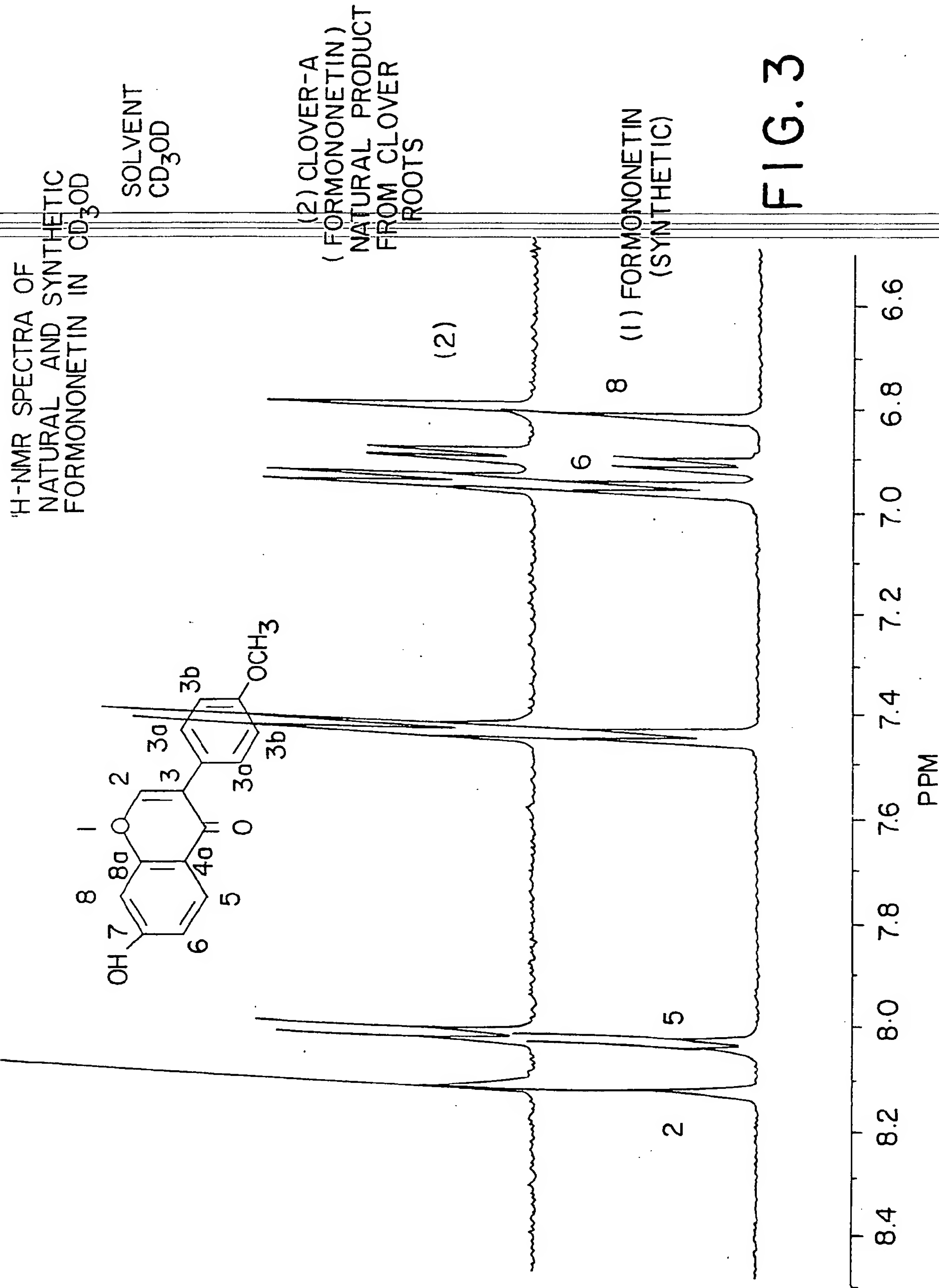
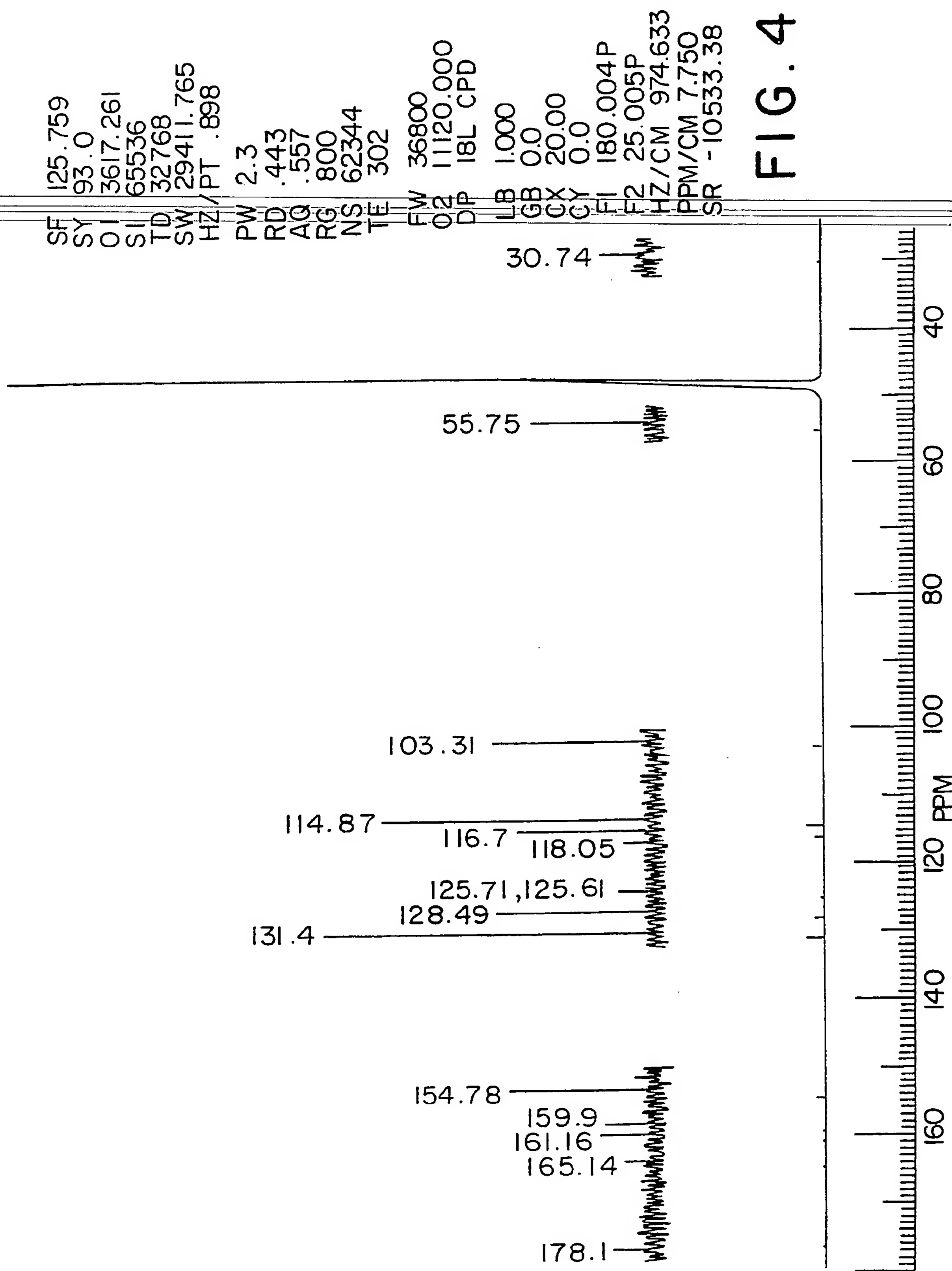


FIG. 1









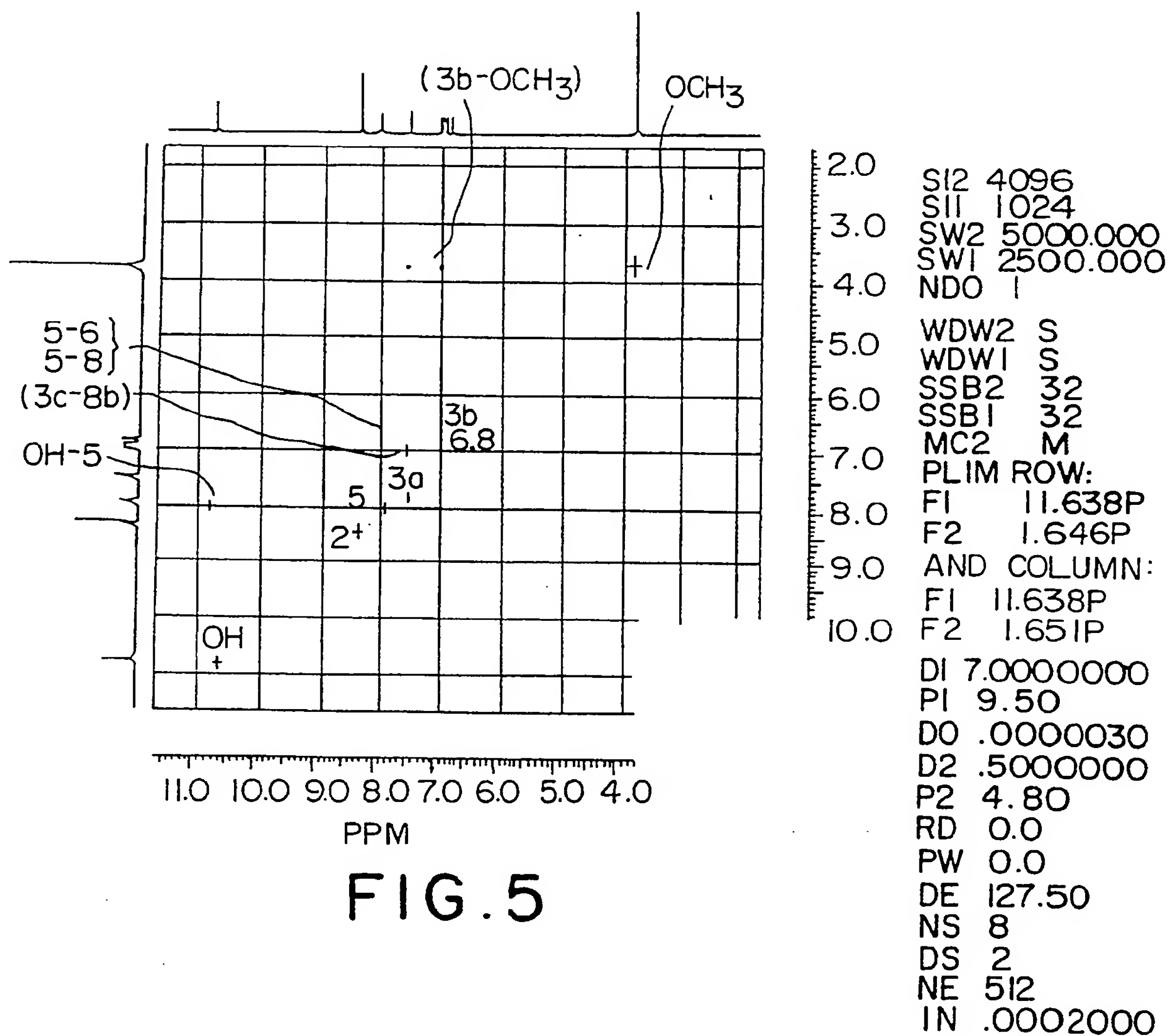
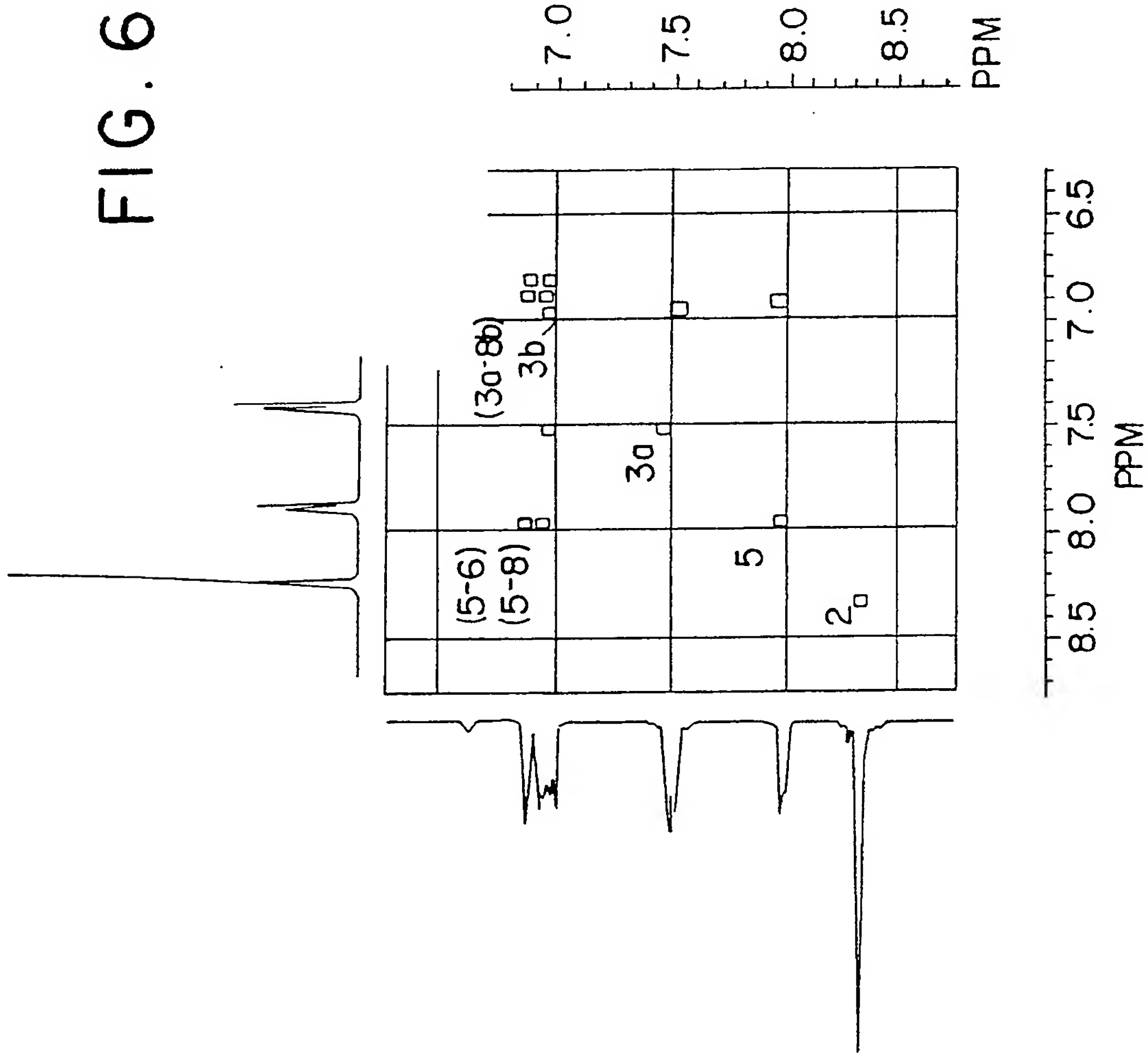


FIG. 5

FIG. 6



SI2 4096  
SI1 1024  
SW2 5000.000  
SW1 2500.000  
NDO 1

WDW2 S  
WDWI S  
SSB2 32  
SSB1 32  
MC2 M

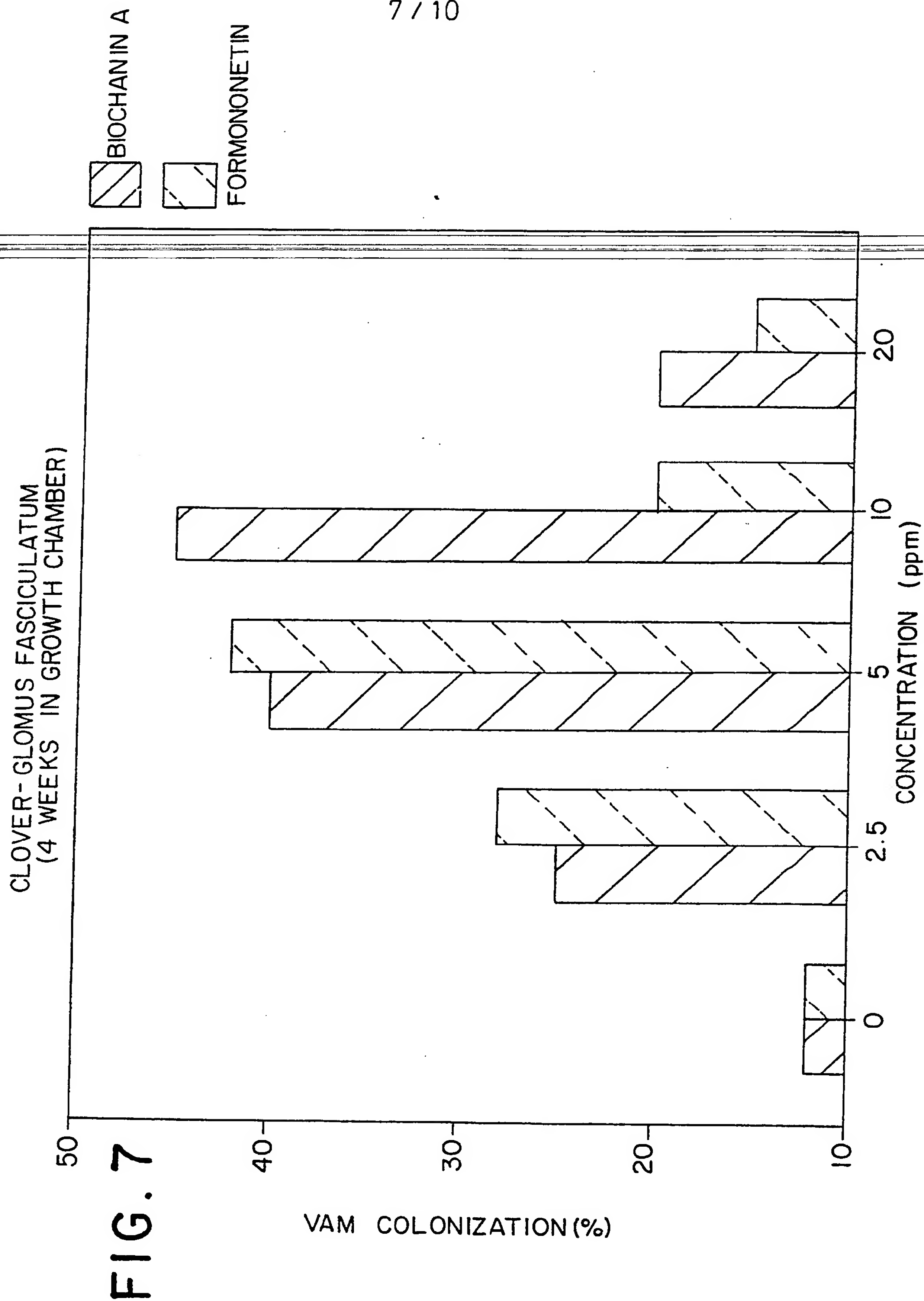
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F1 8.773P  
F2 6.278P  
AND COLUMN:  
F1 8.758P  
F2 6.268P

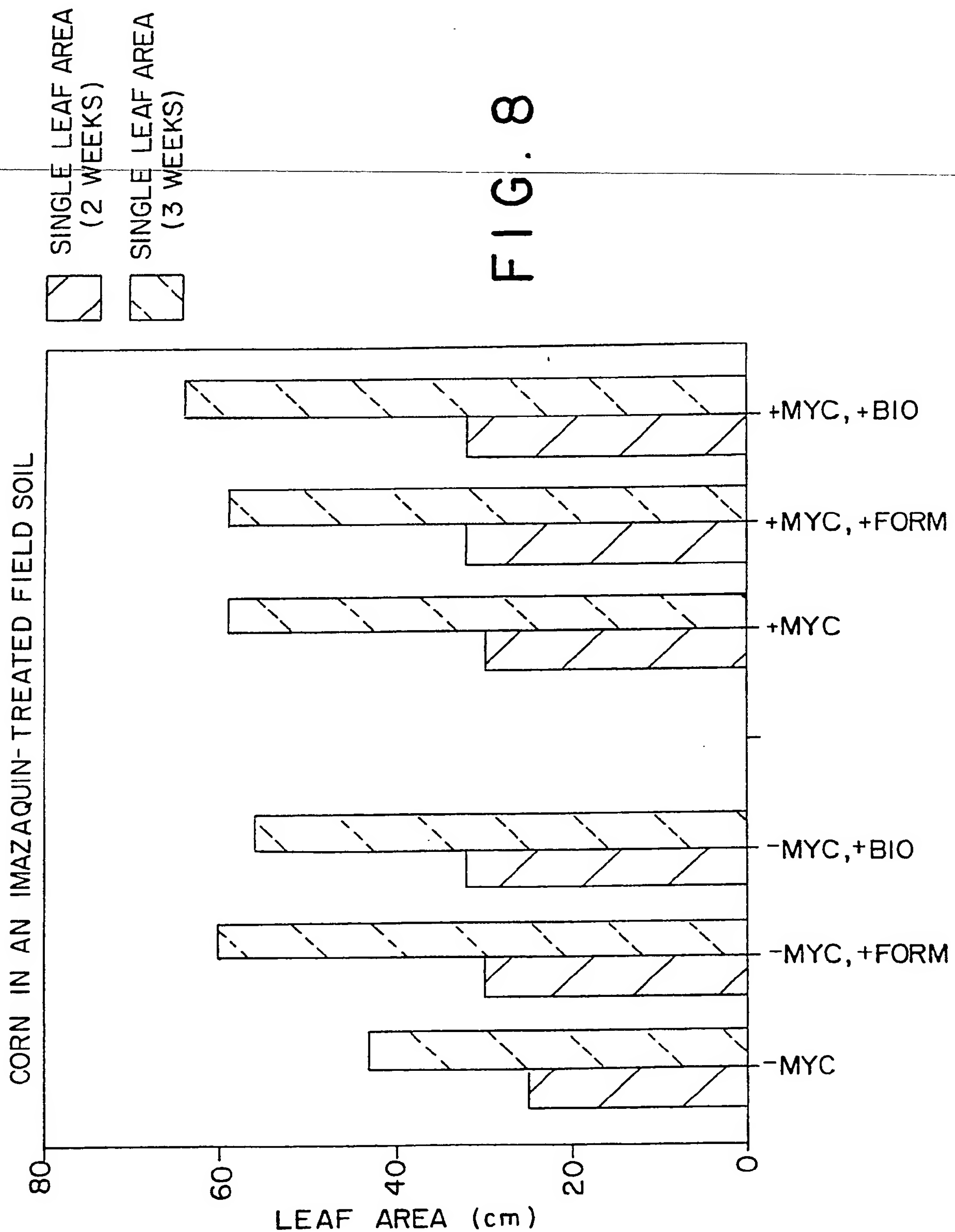
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D2 .5000000  
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RD 0.0  
PW 0.0  
DE 127.50

NS 8  
DS 2  
NE 512

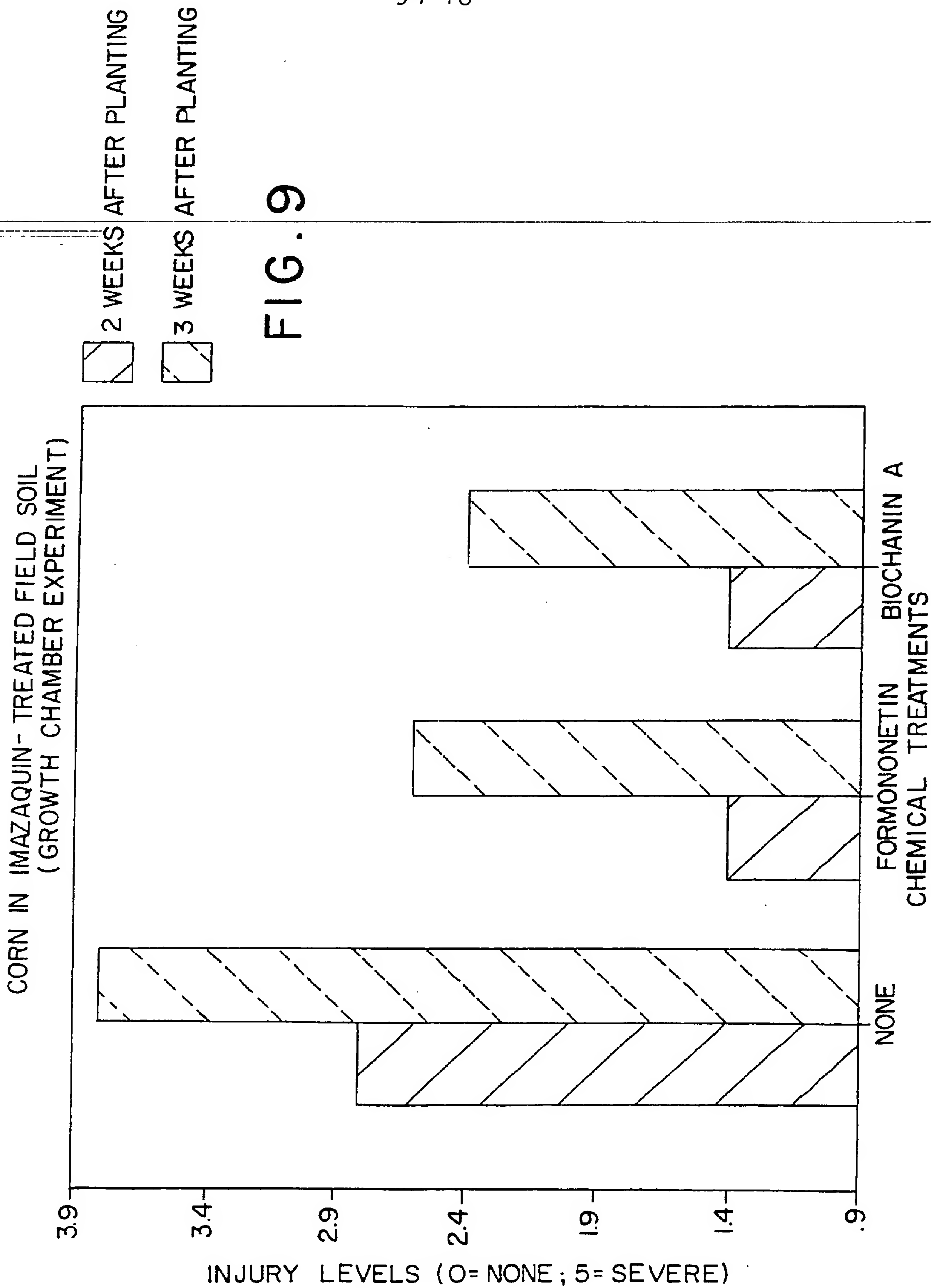
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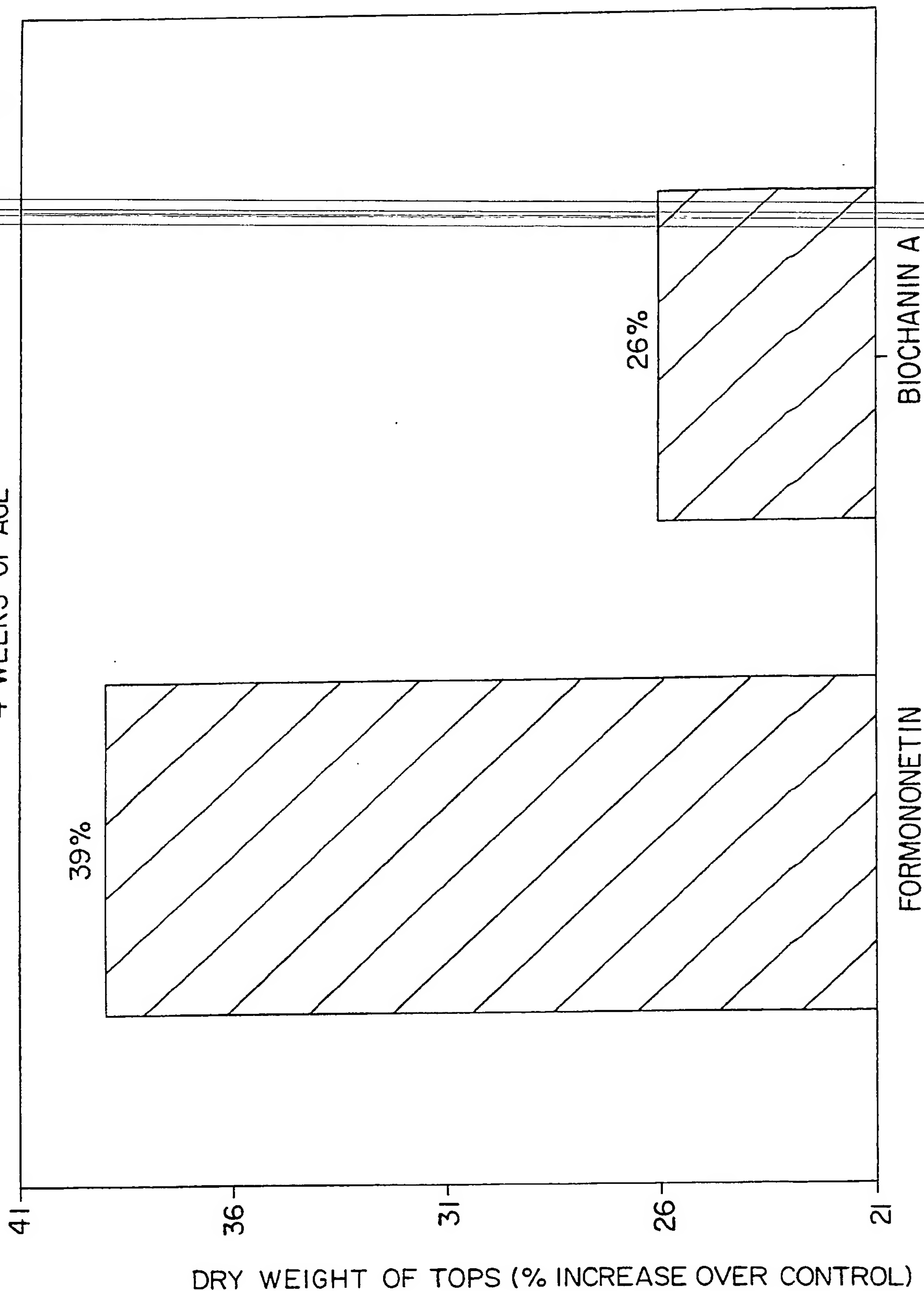




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**FIG. 10**

CORN- IMAZAQUIN TREATED FIELD SOIL  
4 WEEKS OF AGE



## INTERNATIONAL SEARCH REPORT

PCT/US90/06714

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>1</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A01G 1/04; A01C 1/06; C05F 11/08; A01N 65/00, 43/16, 43/50, 43/40, 33/06, 57/00; C07D 311/22 SEE ATTACHMENT		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System <sup>1</sup>	Classification Symbols	
U.S.	47/1.1, 57.6; 514/100; 549/403 71/7, 77, 88, 92, 94, 121	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>6</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>8</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>14</sup>
A	Phytochemistry, 15: 655-659, 1976, H.D. Van Etten, "Antimicrobial Activity of Pterocarpan and other selected Isoflavonoids".	2, 4, 16-18, 20, 21, 29-32, 41, 42
Y	GB, A, 2,043,688 (MOSSE ET AL) 08 October 1980.	1, 3, 8-15, 22-28, 33-35
Y	EP, A, 0,035,296 (PEGASUS PENSION FUND) 09 September 1981.	1, 8-15
Y	US, A, 4,294,037 (MOSSE ET AL) 13 October 1981	1, 3, 8-15, 22-28
A	Annals of Botany, 50: 1-7, 1982, J.C. Steffens, et al, "Molecular Specificity of Haustorial Induction in <u>A. purpurea</u> ..."	1, 2, 4-6, 8-21, 29-32, 36-42
Y	J. L. Harley, et al. "Mycorrhizal Symbiosis", 1983, AP(NY) pp.1-31, 64-103 & 104-116.	1, 3, 5, 6, 8-15, 19, 22-28, 33-40
Y	EP, A, 0,172,085 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE), 24 July 1984.	3, 22-28, 33-35
<p>* Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of this International Search Report <sup>3</sup>	
27 FEBRUARY 1991	22 MAR 1991	
International Searching Authority <sup>1</sup>	Signature of Authorized Official <sup>18</sup>	
ISA/US	S. MARK CLARDY <i>[Signature]</i>	

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim
Y	US, A, 4,551,165 (WARNER), 05 November 1985.	14,15,19
Y	Can. J. Microbiol, 31: 965-972, 1985, J. O. Siqueira et al, "Spores, germination, and germ tubes of vesicular-arbuscular mycorrhizal fungi:"	1,3,5,6,8-15,19 22-28,36-40
Y	US, A, 4,589,225 (STENSAAS), 20 May 1986.	1,8-15
Y	US, A, 4,599,312 (MUENIER ET AL) 08 July 1986.	3,22-28
Y	Phytochemistry, 25: 979-995, 1986 D.A. Smith et al, "Biosynthesis, elicitation, and biological activity of isoflavonoid phytoalexins" (Review Article #16).	2,4-6,16-21,29-32,36-42
Y	Plant Flavonoids in Biology and Medicine, 1986 (Alan R. Liss, Inc.), D.A. Smith et al, "Formation and Biological Properties of Isoflavonoid Phytoalexins." p113-124.	2,4-6,16-21,29-32,36-42
Y	J.A. Bailey. "Biology and Molecular Biology of Plant-Pathogen Interactions," 1986, Springer-Verlag (Berlin), p. 29-36: V. Gianinazzi Pearson, "Cellular Modifications During Host-Fungus Interactions in Endomycorrhizae."	1,8-15
Y	EP, B, 0,209,627 (N.P.I.) 28 January 1987.	3,22-28
Y	Mycorrhizae in the Next Decade (Proc. 7th N. Am. Conf. on Myco.) 3-8 May 1987, C. M. Hepper, "VAM Spore Generation and Hyphal Growth in vitro..." p172-174.	1,2,4-6,8-15,19 22-28,36-40
Y	Applied and Environmental Microbiology, 53: 1928-1933, August 1987, K.S. Elias et al., "Hyphal Elongation of Glomus Fasciculatus in Response to Root Exudates."	3,22-28
Y	Plant Physiology, 84: 1193-1196, 1987, Y. Kapulnik et al, "Flavone Limitations to Root Modulation and Symbiotic Nitrogen Fixation in Alfalfa."	1,2,4-6,8-21,29-32,36-42
Y	CRC Critical Reviews in Biotechnology, 5: 319-357, 1987, P. Jeffries, "Use of Mycorrhizae in Agriculture."	1,3,5,6,8-15,19 22-28,36-40
Y	G.R. Safir, ed, "Ecophysiology of VA Mycorrhizal Plants", CRC Pr(Florida), p.5-25,171-211.	1,3,5,6,8-15,19 22-28,36-40
Y	II Reuniao Brasileira Sobre Micorrizas, 1987, J.O. Siqueira, "Culture axenica e monoxenica dos fungos micorrizicos vesicula-arbusculares" p.44-70.	1,3,8-15,22-28

## CONTINUATION SHEET

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

CATEGORY		RELEVANT TO CLAIMS-NO.
Y	JP, A, 63-87973 (KYOWA HAKKO XOEYO KK) 19 April 1988.	3,22-28
Y	US, A, 4,749,402 (GARRETT ET AL) 07 June 1988.	1,3,8-15,22-28
Y	Science, 242: 346-349, 15 July 1988, M. Jacobs et al, "Naturally Occuring Auxin Transport Regulators."	2,4,16-18,20,21,29-32,41,
Y	J.O. Siqueira et al, "Biotecnologia do Solo: Fundamentos e Perspectivas," December 1988, MEC-ESAL-FAEPE-ABEAS, (Brasilia) p.125-176.	1,3,5,6,8-15,19,22-28,36-
Y	Ann. Rev. Plant Physiol. Plant Mol. Biol. 39: 297-319, 1988, B.G. Rolfe et al, "Genetic Analysis of Legume Nodule Imitation."	1,3,5,6,8-15,19,22-28,36-
Y	Can. J. Bot., 66: 2533-2539, 1988, B. Mosse, "Some studies relating to "independent" growth of vesicular-arbuscular endophytes."	3,22-28
Y	New Phytol. 108: 211-218, 1988, G. Becard et al, "Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots."	3,22-28
Y	New Phytol. 110: 217-225, 1988, M.G. Glenn et al., "Influence of glucosinolate content of Brassica...roots on growth of vesicular-arbuscular mycorrhizal fungi."	3,22-28,33-3
Y	NATO ASI Series, Vol H17, P. Bonfante-Fasolo, "The role of the cell wall as a signal in mycorrhizal associations."	3,22-28,33-
Y	Plant, Cell and Environment, 11: 395-401, 1988, M.D. Templeton et al, "Elicitors and defence gene activation."	1,2-6,8-21,32,41,42
Y	R. Palacios et al, ed. "Molecular Genetics of Plant-Microbe Interactions," May 1988, p.73-91,94-95.	1,8-15

## CONTINUATION SHEET #2

CATEGORYRELEVANT TO  
CLAIM NO.

- |     |   |                                  |
|-----|---|----------------------------------|
| Y   | <del>E.E. Conn, "Recent Advances in Phytochemistry, Vol 22: Opportunities for Phytochemistry in Plant Biotechnology, 1988, p.83-103,113-119,163-175.</del>                  | <del>2,4,16-18,21,29-32,41</del> |
| Y   | Ann. Rev. Plant Physical. Plant Mol. Biol., 39: 221-244, 1988, S.E. Smith et al, "Physiological Interactions between Symbionts in Vesicular-Arbuscular Mycorrhizal Plants." | 1,3,5,6,8-19,22-28,36            |
| Y   | NATO ASI Series, Vol H17, S. Scannerini et al, "Cell to Cell Signals in Plant, Animal and Microbial Symbiosis," p.73-83,167-205.  | 1-6,8-32                         |
| Y   | J.B. Harborne, ed, "The Flavonoids," 1988, Chapman and Hall (London), Chapter 5, "Isoflavonoids" by P.M. Dewick, p. 125-127,137-147,187-192,202-209.                        | 2,4-6,16-21,29-32,36-42          |
| P,Y | New Phytol. 111: 25-39, 1989, A.J.P. Burggraaf et al, "Absence of nuclear DNA synthesis in vesicular-arbuscular mycorrhizal fungi: during in vitro development."            | 3,22-28                          |
| P,Y | US, A, 4,945,059 (OKII ET AL) 31 July 1990.   | 1,3,8-15,22                      |
| P,Y | J. Chem. Ecology, 16: 901-909, 1990, T.L. Wacker et al, "Effects of Ferulic Acid on G. fasciculatum and Associated Effects of Phosphorus Uptake and Growth of Asparagus."   | 1,5,6,8-15,36-40                 |



PCT/US90/06714

CONTINUATION SHEET

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I. CLASSIFICATION OF SUBJECT MATTER

U.S.: 47/1.1,57.6; 71/7,77,88,92,94,121; 514/100; 549/403

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	WO, B, 84/04653 (NATIONAL RESEARCH DEVELOPMENT CORP.), 06 December 1984.	3, 22-28
Y	Physiological Plant Pathology, 24: 357-36, 1984, D. Morandi et al, "Isoflavonoid accumulation in soybean roots infected with vesicular-arbuscular mycorrhizal fungi."	1, 2, 4-6, 8-21, 29-32, 36-42
Y	C.L. Powell et al, "VA Mycorrhiza", 1984, CRC PR (Florida) pp. 1-3, 113-130, 187-203 & 205-223.	1, 3, 5, 8-15, 19, 22-28, 36-40
Y	US, A, 4,550,527 (HALL ET AL), 05 November 1985.	1, 3, 5, 6, 8-15, 19, 22-28, 36-40

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . , because they relate to subject matter<sup>1</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:

3. ☐ Claim numbers . . . , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See Attached Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## ATTACHMENT TO PART VI (form PCT/ISA/210, supplemental sheet)

This International Searching Authority considers that the above-identified international application does not comply with the requirements of unity of invention. Reasons with relevant claims for each invention are:

Group I, Claims 1,3,5,6,8-15,19,22-28,33-40 drawn to a fungal composition, a process of growing VAM fungi, and a method of stimulating plant growth class 71 subclass 7,77,88.

~~Group II, Claims 2,4,16-18,20,21,29-32,41,42 drawn to isoflavonoid compounds and compositions, class 549 subclass 403.~~

Group III, Claims 7,43,44 drawn to a method to alleviate pesticide or herbicide injury, class 71 subclass 94.

Group IV, Claims 7,43,44 drawn to a method to alleviate pesticide or herbicide injury, class 71 subclass 121.

Group V, Claims 7,43,44 drawn to a method to alleviate pesticide or herbicide injury, class 514 subclass 100+.

The inventions listed as Groups I-III do not meet the requirements for Unity of Invention for the following reasons:

Group I alone possesses Unity of Invention as a product, process of manufacture, and method of use.

Group II is an additional product.

Groups III, IV and V are additional methods of use.

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